CHARACTERIZATION AND APPLICATION OF SOME PLANT EXTRACTS FOR CONTROLLING IMPORTANT FOLIAR FUNGAL DISEASES OF TEA

Thesis submitted to the University of North Bengal
for the award of doctor of philosophy
in
Biotechnology

Submitted by
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APRIL, 2014

Declaration

I, Ramashish Kumar hereby declare that the work embodied in my thesis entitled "CHARACTERIZATION AND APPLICATION OF SOME PLANT EXTRACTS FOR CONTROLLING IMPORTANT FOLIAR FUNGAL DISEASES OF TEA" has been carried out by me under the supervision of Dr. Dipanwita Saha, Assistant Professor, Department of Biotechnology, University of North Bengal and Dr. Aniruddha Saha, Associate Professor, Department of Botany, University of North Bengal for the award of the degree of Doctor of Philosophy in Biotechnology. I also declare that, this thesis or any part thereof has not been submitted for any other degree/ diploma either to this or other University.

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This is to certify that the thesis entitled, "**Characterization and application of some plant extracts for controlling important foliar fungal diseases of tea**" submitted by Mr. Ramashish Kumar for the award of the degree of Doctor of Philisophy in Biotechnology is based on the results of experiments carried out by him. Ramashish has worked under my co-supervision at Department of Biotechnology, University of North Bengal and supervision of Dr. Dipanwita Saha, Department of Biotechnology, University of North Bengal. I am forwarding his thesis for the Ph. D. degree (Science) in Biotechnology of the University of North Bengal. He has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in his thesis.

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Acknowledgements

I have much pleasure in expressing my deepest sense of gratitude to my supervisor Dr. Dipanwita Saha, Assistant Professor, Department of Biotechnology, University of North Bengal, for her inspiration, encouragement and guidance throughout the execution of this work. I am also equally grateful to my co-supervisor Dr. Aniruddha Saha, Associate Professor, Department of Botany, University of North Bengal for his guidance and advice during my research work.

I am also indebted to Dr. Ranadhir Chakraborty (Head) Department of Biotechnology, University of North Bengal, Dr. Shilpi Ghosh and Dr. Anoop Kumar of the Department. I extend my special gratitude and respect to Prof. B. Basu, Dr. P. Ghosh and Dr. A. K. Nanda of the Department of Chemistry for their help. I also want to thank Head, Department of Chemistry, University of North Bengal for providing instrumental facilities. Thanks are also due to Dr. Runu Ghosh, Scientist, Department of Biotechnology, University of North Bengal.

I am thankful to Mr. Jayanta Das, Senior research fellow, Dept. of Chemistry for helping in spectral data analysis and structure elucidation. Regards are also due to the Head, SAIF, Central Drug Research Institute, Lucknow and SAIF, NEHU, Shillong for providing the service of NMR and ESI-MS of my samples. Thanks are also due to the authorities of different Tea

Estates of Darjeeling Districts of West Bengal with special reference to Gaya Ganga Tea Estate.

I express my thanks to my colleagues, Ms. A. Ghosh, Ms. Gargee Dhar Purkayatha, Ms. Sima mandal, Mr. R. Dey Sarkar, Ms. S. Bhowmick, Mr. A. Kumar, Mr. K.K. Singh, Mr. B. Tiwari and Mr. A. Mandal of the Dept. of Biotechnology, University of North Bengal and Dr. Sourish Das Gupta, Mr. H. K. Barman, Mr. S. Ghosh, Mr. B. Saha, Mr. H. Mandal, Mr. S. Das, Mr. P. Chakraborty, Ms. P. Sarkar and Mr. A. Saha of the Dept. of Botany, University of North Bengal.

I express my heartfelt gratitude and respect to my beloved parents, Mrs. Malti Devi and Shree Bhagawat Prasad for their endless encouragement, inspiration and support. Special thanks are due to my wife Mrs. Surbhi Suman for her continuous support and help throughout the execution of the work.

Lastly, I must thank Indian council of medical research (ICMR) for providing research grant and fellowship to me.

(Ramashish kumar)

ABSTRACT

Tea is the most widely consumed hot beverage in the world today. It is a major plantation crop and very important contributor to the economy of north east India, as a considerable size of population is dependent on tea for their livelihood. Tea leaves are susceptible to attacks by foliar fungal pathogens that result in diseases and major crop loss. The important foliar fungal pathogens are thus to be controlled so as to reduce disease incidence in plants. The use of synthetic chemical fungicides is unpopular due to residual toxicity, non targeted environmental impacts and direct or indirect effects on animal health system. Therefore, an eco-friendly approach to control plant disease is necessary. Plant products are safer in comparison to synthetic fungicides, as they have no deleterious effect on environment and on non target organisms in nature. Plants usually produce diverse group of secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which are of great importance to human and plants.

The present work aims towards appropriate utilization of phytoextracts for controlling diseases in tea plants. It is always important that the pathogens of crop plants are identified correctly for designing appropriate control strategies. DNA based approaches are now common in the identification of plant pathogens. However, literature study on pathogens of tea showed that such modern approaches have been used by very few authors in identification or characterization of the fungal pathogens. Hence, it was considered worthwhile to study the etiological aspects associated with blister blight disease. The pathogen of the disease is a biotroph, popularly known as *Exobasidium vexans*, which is considered as the most dreaded among tea pathogens. Therefore, the basic objectives of the present study are: (1) Isolation and molecular characterization of fungal pathogen from infected tea plants. (2) Screening of

tea varieties from Darjeeling Hills and adjoining plains for susceptibility of a pathogen by ELISA. (3) Screening of potential plant preparation for controlling major tea diseases. (4) Isolation and phytochemical characterization of antifungal molecules involved in disease control, (5) Formulation of useful product which may be applicable as bio-fungicides in susceptible tea plants.

To fulfill the objectives, blister blight affected leaf samples were collected from tea plants from tea gardens of Darjeeling district to study the etiology of the disease. Six fungal strains were isolated from the samples that were cultured in PDA. The fungal isolates were characterized morphologically through microscopic observation and phylogenetically by 18S rRNA gene sequencing. For identification of unculturable endophytes, part of the 18S rRNA gene of the fungus was amplified by PCR using plant genomic DNA as template with fungal universal primers (ITS-1 and ITS-4) and the amplified products were cloned in pGEM T- easy vector. The positive clones were screened for presence of inserts by PCR and the recombinant clones were screened further by RFLP analysis using *Cfo*I. The clones with distinct restriction band patterns were identified and subjected to sequencing. All sequences were used for similarity searches through the BLAST function of GenBank. Results showed that the strains belonged to the genera *Exobasidium*, *Colletotrichum*, *Sporobolomyces*, *Fusarium* and *Neofusicoccum*. Several sequences matched up to 99% with unnamed uncultured endophytic fungal sequences submitted in the GenBank. All sequences were deposited in NCBI GenBank through BankIt and Accession Numbers were provided for each of the strains. Phylogenetic relationship was drawn for *Exobasidium* sp., the causal agent of blister blight, which revealed that this fungus clustered with *E. reticulatum* strains. A particular strain of *Colletotrichum gloeosporioides* CR5 was identified as pathogen (brown blight) after verification of Koch's postulates.

Pathogenicity of CR5 was determined in eight clonal varieties of tea by detached-leaf assay. The varieties TV-30 and TV-18 were considered susceptible

while TV-25 and TV-9 were resistant after 72 hrs inoculation. Pathogenicity of the isolate was further confirmed by determining the levels of cross reactive antigens by indirect ELISA. Antiserum against CR5 mycelial antigen was raised in rabbits and tested for crossreactivity against tea leaf antigens at a concentration of 20µgml^{-1}. Cross reactive antigens were detected at high levels between pathogen and susceptible tea varieties and at lower levels when resistant varieties were used. Result showed that TV-18 and TV-30 varieties are more susceptible than other six varieties. This clearly indicated that cross-reactivity was higher between pathogen and susceptible variety than between pathogen and resistant variety.

In order to control foliar diseases by botanicals, fourteen plants were selected based on previous reports of medicinal properties and availability in the Sub-Himalayan region of West Bengal. Selected plants were screened for their antifungal potential against four different fungal pathogens of tea by agar diffusion technique. Antifungal component(s) in a plant was detected by TLC bioautography method. The active components were isolated and purified by repeated column chromatography following bioassay guidance. Altogether thirteen compounds were characterized and their structures were elucidated by UV, IR, ^{1}H & ^{13}C 1D and 2D NMR and ESI-MS spectroscopy data analysis. Compounds belonging to several classes such as furanocoumarin, coumarin, sesquiterpene, clerodane diterpene and phenolics were purified from different plant extracts. Excavarin-A and excavatin-I were isolated from leaves of *Clausena excavata.* Another five compounds, clausenidin, clausarin, dentatin, nordentatin and osthol were isolated from root bark of this plant. 8-*epi*-xanthatin and 8-*epi*-xanthatin-1ß,5ß-epoxide was purified from *Xanthium strumarium* leaf extracts. Compounds, 16-Oxocleroda-3,13 (14)E-dien-15-oic acid, chavibetol acetate and eugenol were isolated from *Polyalthia longifolia*, *Piper betle* and *Ocimum sanctum* respectively. Minimum inhibitory concentrations of antifungal compounds were determined against several fungal phytopathogens by microdilution bioassay. Compounds, excavarin-A, excavatin-I, osthol, 8-*epi*-

xanthatin, 8-*epi*-xanthatin-1ß,5ß-epoxide, 16-Oxocleroda-3,13(14)E-dien-15-oic acid, chavibetol acetate and eugenol showed broad antifungal activity against both plant and human fungal pathogens. The purification of a new antifungal coumarin, excavarin-A from leaves of *C. excavata* extract was reported during this study. Other biological activities like antioxidant and oxidative DNA damage protective activities were also tested in compounds which failed to show antifungal activity. The compounds, clausarin and nordentatin showed strong antioxidant and DNA damage protective activity. The antifungal activity of 16-Oxocleroda-3,13 (14)E-dien-15-oic acid was increased by allylic hydroxylation at C-18 position following biotransformation process using the fungus *Rhizopus stolonifer*. This derivatization resulted in the purification of another new compound 18-hydroxyl,16-oxocleroda-3,13 (14)E-dien-15-oic acid with strong antifungal property.

Finally, selected antifungal compounds as well as crude extracts were tested for their efficacy to control brown blight disease in tea. Emulsified formulations of the extracts were used for determination of *in-vivo* MIC in detached leaves of tea. Concentrations of extracts that were slightly higher than MIC was used for spray treatment for controlling experimentally induced brown blight in tea plantlets in the green house.

Treatment of tea plantlets with formulations made from purified compounds of *X. strumarium* and *C. excavata* at 2 mg ml^{-1} concentration reduced the disease incidences significantly ($P<0.05$) when compared to control sets. Disease incidence was reduced by 90.4% by 8-*epi*-xanthatin; by 87.6% by 8-*epi*-xanthatin-1ß,5ß-epoxide and by 89.7% by excavarin-A in TV-30 variety. Similarly, the plants treated with crude extract formulation also showed high percentage (76%) of reduction in disease incidence. The purified preparation compared favourably with the synthetic fungicide bavistin whereas the crude extract was slightly less effective. When observed for phytotoxicity, both pure and crude extract formulation did not show any visible phytotoxicity upto 12 days of

spraying. A study on the effects of the extracts on rhizosphere and phylloplane bacterial populations showed that 84% of the bacteria from the phylloplane and 88% of the bacteria from the rhizosphere were resistant to *C. excavata* crude extracts. However, only 62% of the rhizosphere bacteria were resistant towards *X. strumarium* extracts at the tested concentrations.

In the present study, several bioactive compounds were purified and some were found to be effective in controlling foliar fungal diseases of tea. Considering the high antifungal activity and low phytotoxicity of phytochemicals isolated from *X. strumarium* and *C. excavata* extracts under the test conditions, the extracts may be available for the control of various tea leaf diseases after appropriate field tests. The findings of this study may be significant to the tea industry as an increasing number of tea gardens are now shifting to organic farming methods.

PREFACE

In the last 50 years, the agriculture and crop protection industry has seen the progressive development and application of technology coupled with better chemical innovation that has led to dramatic increase in crop production. Driven by consumer and environmental safety concerns, the regulatory framework for risk assessment and risk management of plant protection products have been refined at the administrative level in many developed and developing countries. During this period, increasing sophistication of crop protection agents to guarantee economic yields and to provide the quality, demanded by consumer markets has raised many challenges to food-safety and has put forth several issues around the frequently asked question — how safe is our food?

An industry in India which depends heavily on pesticide use is tea production. Tea is the most widely consumed non-alcoholic caffeine containing beverage in the world. The health benefits of green tea include alleviation of a wide variety of ailments, such as different types of cancer, heart disease and liver disease. The tea plant, however, is itself disease prone, mainly due to invasive fungal pathogens which attack almost all parts including the harvestable two-and-a-bud region. It has been estimated that 67 million pounds (30 million kg) of tea is lost per annum due to pests and fungal diseases in northeast India. Therefore the growers are forced to rely on huge amount of chemical fungicides for ensuring at least a moderate production.

The chemical inputs to agriculture have contributed substantially to the spectacular improvements in crop productivity and quality over the past 100 years. However, the damage to the environment caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some pesticide-adversaries have led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Apart from strict regulations on chemical pesticide use, there is added political pressure to confiscate the most hazardous chemicals from the market. Tea-decoction is prepared directly from the

processed leaves and drank as a beverage. Consumers may be directly exposed to pesticides present as residues in the drink. The protection of health of consumers, users and the environment is the driving principle behind disease control by environment-friendly ways. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. These alternatives may be in the form of botanicals or chemicals of plant origin.

Some plants have been found to naturally produce compounds that prevent the growth of fungi. These are basically defense-related secondary metabolites which belong to several classes such as phenolics, coumarins, terpenoids, alkaloids and peptides. These compounds may be extracted to replace the synthetic chemicals as fungicides. Being natural products, these phytochemicals are considered safe and benign to the environment. The in-built advantage of using natural compounds in agriculture is that they are non phytotoxic, easily biodegradable and less detrimental to non target organisms.

The work embodied in this thesis was initiated in the year 2009 with broad objectives of controlling tea diseases by utilizing the plant products that are antifungal and non-phytotoxic. The status of the work and their results and inferences drawn thereof is presented in three major chapters and additional supplementary details given as appendix at the end.

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ABBREVIATIONS

A_{260}	Absorption at 260 nm
A_{280}	Absorption at 280 nm
AS	Antisera
AA	ascorbic acid
BSA	Bovine Serum Albumin
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
^{13}C NMR	Carbon nuclear magnetic resonance
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
COSY	Corelation spectroscopy
CD	Critical difference
d	days
^{o}C	Degree Celcius
$dNTP_{Mix}$	Deoxynucleotide triphosphate mix
DNA	Deoxyribonucleic acid
dia	diameter
DI	Disease Index
DEPT	Distortionless enhancement by polarization transfer
ELISA	Enzyme linked Immunosorbent Assay
EtOH	Ethanol
EtoAc	Ethyl acetate
EDTA	Ethylene diamine tetra acetate
$FeCl_3$	Ferric chloride
GIS	Geographic information system
HETCOR	Hetronuclear correlation
h	hours

HCl	Hydrochloric Acid
^{1}H NMR	Hydrogen nuclear magnetic resonance
H_2O_2	Hydrogen peroxide
IARI	Indian Agricultural Research Institute
ICAR	Indian Council of Agricultural Research
ITS	Internal Transcribed Spacer
IPTG	Isopropyl-ß-D-1-tiogalactopyranoside
kb	kilobase
kg	kilogram
LB	luria bertani
MeOH	Methanol
MTCC	Microbial type culture collection
µg	microgram
µl	microlitre
µm	micrometer
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
mmol	millimole
MIC	Minimum inhibitory concentration
Min.	Minute
M	molar
ng	nanogram
nmol	nanomole
N	Normal
NBU	North Bengal University

No.	Number
ocDNA	Open circular DNA
OD.	Optical density
Pet ether	Petroleum ether
pmol	picomole
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
$R_{f.}$	Relative front
RNase	ribonuclease
RM	Richard's Medium
rpm	Rotation per minute
S	seconds
SDS	sodium dodecyl-sulphate
sp.	Species
scDNA	Supercoiled circular DNA
T.E.	Tea Estate
TLC	Thin layer chromatography
Tris	tris (hydroxymethyl) amino methane
Tris-HCl	tris (hydroxymethyl) amino methane-hydrochloric acid
TE	Tris- EDTA
TAE	tris-acetate ethylene diamine tetra acetat
TE	tris-hydrochloric acid and ethylenediaminetetra acetate
UV	ultraviolet
vol	volume
v/v	volume by volume
w/v	weight by volume

CONTENTS

1.1. Tea: An overview

The most widely consumed and the cheapest hot beverage in the world today is tea (*Camellia sinensis* (L.) O. Kuntze). It belongs to the family Theaceae. The cultivated taxa comprise of three main natural hybrids. They are *Camellia sinensis* (L.) O. Kuntze or China type, *Camellia assamica* or Assam type, and *C. assamica* sub sp. *lasiocalyx* (Planchon ex Watt.) or Cambod type. Tea is made from the young leaves and unopened leaf buds of the tea plant, *Camellia sinensis* (L.) O. Kuntze. The tea growing in different countries can be classified into three types, namely, black, green and oolong tea. Freshly harvested tea leaf is processed and consumed differently in different parts of the world, 78% black, 20% green and less than 2% oolong tea (Kuroda and Hara, 1999). It is one of the most important plantation crops in India and is extensively cultivated in the North Eastern states as well as the Deccan Plateau region. While cultivated in various countries, South East Asia is the original home of tea and China is the first country to use tea as beverage (Barua, 1989). Black tea is consumed primarily in Western countries and in some Asian countries, whereas green tea is consumed primarily in China, Japan, India and a few countries in North Africa and the middle East North-East India, South India and Himachal Pradesh (Uttaranchal). In North-East India, tea is cultivated largely in Assam and West Bengal (Darjeeling). It is cultivated as a plantation crop, grows well in acidic soil and a warm climate with at least 50 inches of rain per annum. Tea's flavors, quality and character are completely dependent on the region, the altitude, the type of soil and the climate in which it is grown. The world famous British East India Company started commercial production of tea in India and converted massive tracts of land to its production.

1.2. Economic importance

Tea production as an agro-based industry is a very important contributor to the economy of India. The main tea business in India is located along the rural hills and backward areas of northeastern and southern states like Assam, West Bengal,

Tamil Nadu and Kerala where a considerable size of population is dependent on tea for their livelihood. India occupies 1.016 million acres of tea growing land which is 16.4% of the total tea growing areas of the world. India is the single largest producer of tea and about 30% of the world's tea produced in India comes from Assam, South India, Kangra and Uttar Pradesh. India exports more than 28% of the world's tea (Singh et al., 2006).

1.3. Health benefit

Tea is an aromatic beverage usually prepared by pouring hot or boiling water over cured leaves of the tea plant. It originated in China as a medicinal drink. Today tea is the most consumed hot beverage in the world after water (Cabrere et al., 2006). Green tea consumption as herbal drink is helpful in maintaining and promoting health (Mckay and Blumberg, 2002) including the prevention of cancer (Fujiki, 2005), cardiovascular diseases and anti-inflammatory (Sueoka et al., 2001), antibacterial (Sudano et al., 2004), antioxidant (Osada et al., 2001) antiviral (Weber et al., 2003), neuroprotective (Weinreb et al., 2004) and cholesterol-lowering effects (Raederstorff et al., 2003). Besides, tea leaves contain more than 700 different chemicals including flavanoides, amino acids, vitamins, caffeine and polysaccharides which are associated with cell-mediated immune responses of the human body, protection against intestinal disorders and overall health damages (Chen, 1999; Islam et al., 2005).

1.4. Diseases and pathogens

Tea leaves are harvested several times in an every year, about at intervals of 5-7 days. With the introduction of different improved varieties and expansion of plantation to newer areas, disease-related problems have increased manifolds. The plant is susceptible to attack by a number of foliar pathogens which causes diseases. Additionally, the varying conditions of climate, soil and several environmental stresses under which the plants are cultivated makes them prone to a wide variety of pathogen attack. Unlike the bacterial, algal and viral pathogens, the fungal pathogens pose significant threat to tea cultivation (Saha

et al., 2001; Chakraborty et al., 2009; Saikia et al., 2011). The most important leaf and root diseases affecting tea plants in North East India are blister blight, black rot, red spot, grey blight, brown blight, charcoal stump rot, brown rot, black root rot, violet root rot and diplodia (Saha et al., 2008; Barthakur, 2011). The fungi attack mainly during persistent high humid condition, which is very common in the tea growing regions arising out of a longer rainy season and thereby creating perfect conditions for the pathogens to proliferate. The major foliar fungal pathogens are *Exobasidium vexans, Colletotrichum gloeosporioides* (anamorph) = *Colletotrichum camelliae, Pestalotiopsis theae, Corticium invisum, Corticium theae* etc. (Sarmah, 1960). Blister blight (*Exobasidium vexans*), which attacks young leaves and shoots, is the most serious fungal diseases of tea in North-East India (Satyanarayana and Baruah, 1983) and all tea growing areas of Asia (Arulpragasam, 1992). The net blister blight is a causative agent *Exobasidium reticulatum* is also reported mainly in Japan and Taiwan. Brown blight caused by *Guignardia camelliae* (Cook) butler (asexual stage=*Colletotrichum camelliae* Massee) is the most common leaf disease of tea in China was reported (Chen and Chen, 1982). Besides these major pathogens, several new reports of diseases affecting tea plants of different ages and varieties are recorded. These are caused by *Curvularia eragrostidis*, *Bipolaris carbonum* etc. (Chakraborty, 1987; Saha et al, 2001). Several varieties, some of which are elite varieties are found to be more susceptible to different pathogens than others (Dasgupta et al, 2005).

1.5. Control strategy

Since tea plants are susceptible to attack by severe foliar fungal pathogens; therefore, tea cultivation requires the use of large amount of chemical fungicides (Singh, 2005). In the past, farmers have used synthetic fungicides for controlling fungal diseases of crop and improvement of crop productivity, but they have now become unpopular due to several reasons, such as residual toxicity (Seth et al., 1998), non target environmental impacts, growing cost of pesticides, modified safety regulations (Gerhardson, 2002; Compant et al.,

2005), development of pathogen resistance (Van den Bosch and Gilligan, 2008) and directly or indirectly affect animal health (Begum et al., 2007).

The alternative source is the use of botanical extracts as fungicides to control fungal infection and detection of new antifungal compounds which have no side effects on environment or on animal health (Saha et al., 2005a, 2005b). Plant products are safer to environment as compared to synthetic fungicides, which has no deleterious effect on environment and on non target organisms in nature. The major characteristics of such bio-rational pesticides are that they should have minimal toxic effects on human and other non-target organisms and rapid degradation ability (Abou-Jawdah et al., 2004). Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which are of great importance to humans and plants. Useful antimicrobial phytochemicals are phenolics, terpenoids, essential oils, alkaloids, coumarins, lectins and polypeptides (Dahanukar et al., 2000). Several authors have reported antifungal activity of plant extracts against pathogens of rice (Lalitha et al., 2011), tomato (Ravikumar and Garampalli, 2013), wheat (Schalchli et al.,2012), pea (Al-Askar and Rashad 2010) etc. However such report involving tea pathogens are very few (Chakraborty et al., 1991; Saha et al., 2005a, 2005b; Saha et al., 2012). Exploitation of naturally available chemicals from plants, which retard the reproduction of undesirable microorganism, would be more realistic and ecologically sound method for protection of tea plants against diseases. Thus plant based secondary metabolites, which have defensive roles, may be exploited for the management of foliar fungal diseases.

1.6. Objectives

Development and effective adoption of disease control strategies requires a greater understanding of the intricate interactions among pathogen, plant products and the environment. The research presented here aims towards appropriate utilization of phytoextracts in limiting fungal diseases of crops. The

accurate identification of any organism causing diseases in crop plants is of great importance for determining appropriate control strategies. Molecular approaches are commonly used in the identification of pathogens but a literature study reveals that such modern approach have not been utilized in identification or characterization of pathogens of tea. Hence, it was considered worthwhile to study the etiological aspects associated with blister blight disease popularly known to be caused by the biotroph *Exobasidium vexans*, the most dreaded foliar pathogen of tea. Therefore the basic objectives of the present study are

1. Isolation and molecular characterization of fungal pathogens from infected tea plants.
2. Screening of tea varieties from Darjeeling Hills and adjoining plains for susceptibility towards the pathogen by ELISA.
3. Screening of potential plant preparation for controlling major tea diseases.
4. Isolation and characterization of antifungal molecules involved in disease control.
5. Formulation of useful product which may be applicable as bio-fungicides in susceptible tea plants.

2.1. Literature review:

The growth and productivity of a plant is adversely affected by external invasions caused by pathogenic microorganisms that lead to diseases. Diseases are considered as a major factor that limit crop yield. Plant disease may be defined as the result of a dynamic, detrimental relationship between the organism that parasitizes or interferes with the normal processes of cells or tissues or both of the plant. The organism that causes the disease process within the host is called a pathogen. The pathogens in general interferes with plant cell functions by producing toxins that damage cells or do not allow the normal growth and multiplication of plant cells. Pathogenicity refers to the ability of a pathogen to interfere with one or more cell functions within the plant system. Virulence describes how well or the rate at which the pathogen is able to interfere with the cellular machinery (Ptaky and Carson, 2004). A virulent pathogen can be very aggressive and incite disease under a wide range of environmental conditions; in contrast an avirulent pathogen rarely interferes with cell functions or may do so only under very specific environmental conditions or when the plant is debilitated by other causes. Tea plant is a woody perennial and as such is exposed to a wide range of environmental stresses throughout its life-span which may predispose the plants to pathogen attack.

2.1.1. Diseases of tea

Tea, being an economically beneficial crop, is often forced to grow under varying climatic and soil conditions remote from its natural environment. The profit associated with tea cultivation has also made it a subject of various cultural treatments which are widely in variance with its natural conditions for growth. Tea is a plantation crop which requires the growing of the perennial plant in a pure stand extending over a period of about 40-100 years and over vast areas which affords a happy hunting ground for pests and diseases of all kinds. Microbial diseases of tea can be caused by one or more of several agents majority of which

belong to the kingdom fungi and few are bacteria and algae. Fungi alone account for almost all the major recognized diseases of tea. All parts of the tea plant of all ages are vulnerable to pathogen attack. Agnihothrudu (1964) listed 385 species of fungi which were reported to affect different parts of the tea plants. After a thoroughly revision, Chen and Chen (1989) reported that a total of 507 fungi had been recorded in tea. Barua (1989) accounted that out of the 385 species of fungi reported as tea pathogens from all over the world, about 190 were found to be associated with tea in North-East India.

2.1.2. Blister blight

Blister blight occurring on tea leaves caused by *Exobasidium vexans* Massee is regarded as one of the most common and damaging diseases of tea of the Darjeeling hilly regions (Lujaerajumnean and Tummakate, 1987; Barthakur, 1984). The disease attacks young succulent growth that includes the tender leaves and stem of tea bushes which is plucked for processing thereby directly affecting the harvest (Singh et al., 2006). The disease is prevalent throughout North-East India during autumn and spring and during rains in the hilly regions. Severe outbreaks often occur in favourable climate in moist, cold and misty conditions that helps in rapid propagation of the air-borne spores (Sarmah, 1960; Lujaerajumnean and Tummakate, 1987).

A single spore can produce a blister when deposited on tender shoots in about 10-21 days from the time of infection. The fungus produces pale yellowish circular spots upto 12 mm diameter which are glistening and concave on the upper surface and white or pink, powdery convex on the underside. Blister coalescence induces leaf distortion and curling (Singh et al., 2006).

The spores may be carried by the wind and fog to long distances. However, they do not remain viable for long in dry hot weather (Sarmah, 1960). The pathogen is extremely sensitive to sunlight and requires high humidity or free water on the leaf surface for germination. Epidemics of the disease are therefore

common in high altitudes when the climate is humid and foggy. The fungus is primarily a biotroph and mycelia do not grow under laboratory conditions.

Blister blight is capable of causing enormous crop loss throughout the tea growing regions of Asia, especially in India, Sri Lanka, Indonesia and Japan. It is one of the earliest known leaf diseases of tea. Blister blight caused by *Exobasidium vexans* Massee was first reported in Assam, India in 1868 and suddenly appeared in the Darjeeling district (India) in 1908 and consequently it became the major problem of all tea plantations of Asian Countries (Lujaerajumnean and Tummakate, 1987). It was also recognized in Japan in 1920. In a study in Sri Lanka, a crop loss of 33% was recorded in unprotected areas compared to fields which were sprayed with chemicals (de Silva et al., 1977). Since the pathogen attacks harvestable tender shoots, it inflicts massive yield loss of 40% and quality deterioration is noticed even below the 35% disease threshold level (Gulati et al., 1993). An enormous crop loss amounting to 50% of the total annual crop has been reported for unprotected areas from South India (Venkata Ram, 1968).

Baby (2002) reported that blister blights are most destructive foliar disease of tea, caused by obligate parasitic fungus *E. vexans*. The disease is known to occur in almost all tea-growing areas of Asia, but it is most serious in India, Sri Lanka, Indonesia and Japan. Blister infections in tea plant affect the quality and quantity of the harvested leaves. Because leaves are harvested and dried to make tea products, this pathogen can cause substantial losses in yield and quality. It spreads and infects the crop in southern and eastern Asia during seasons of high humidity.

Chen and Chen (1982) reported blister blight occurring in Japan as Japanese *Exobasidium* blight caused by *E. reticulatum* Ito & Sawada which occurs on mature leaves. The authors pointed that the disease may be distinguished from blister blight by the white dust-like network of fruit bodies of the causal fungus on the underside of diseased leaves. Singh et al. (2006) referred to the disease caused by *E. reticulatum* as 'Net blister blight' which, according to the authors, is

reported from Japan and Taiwan only. They described that the infected leaves develop white reticulate lesions on the under surface which are slightly protuberant along the veins. The infected leaves may fall off and petioles may die back during severe out breaks of the disease (Singh et al., 2006).

2.1.3. Brown blight

Another important leaf disease of tea is brown blight which is extremely common in on old leaves of tea bushes throughout North-East India that are about to fall and also found on leaves of all ages when the plant is weakened by other causes such as severe attack of red spider, lack of nitrogen, water logging, sun-scrotch, hail damage, drought, lack of shade etc. (Sarmah, 1960). It is reported as the most common leaf disease of tea in China (Chen and Chen, 1982). The symptoms usually appear at the margin of the leaves as brown patches which gradually spread inwards. The edges of the patches are sharply defined and more often marked with a delicate concentric zonation. The disease is favoured by poor air circulation, high temperature and high humidity or prolonged periods of leaf wetness. When young twigs of susceptible cultivars are cut and used to root new plants, latent mycelium in the leaf tissue may start to invade nearby cells to form brown spots and this may lead to death of leaves and twigs. The disease is mostly found to occur along with grey blight (*Pestalotiopsis theae*) and often observed together in the same leaf (Sarmah, 1960; Keith et al., 2006).

2.1.4. Other tea diseases

Gray blight is a foliar fungal disease of tea caused by *Pestalotiopsis theae* Sawada. The disease symptoms are generally circular, with upper surface concentrically zoned with different colors (Chen and Chen, 1982). It has been reported from all tea growing countries of the world that the disease appears on bare stalk and young plants as well as in mature leaves (Baby and Sanjay, 2006).

Black rot is an important disease of the mature leaf and stem caused by fungi *Corticium theae* and *Corticium invisum.* They attack the maintenance

leaves, causing gradual deterioration in the health of the bush and consequent loss of crop. The infected leaves turn black as they rot during the wet weather. The dead leaves, which are detached from the bush but remain suspended on the bush and the mycelial chords come out from the infected leaves hold the bush. Then the fungal sclerotia embed themselves in the cracks and crevices of the stem and bark (Chen and Chen, 1982; Singh et al., 2006).

Alternaria alternata, is also a foliar fungal pathogen of tea in North Bengal, India. Disease symptoms first appear as greyish brown patches around tips and margins of young leaves which extend towards the midrib resulting in leaf curl, death and defoliation (Chakraborty et al., 2005).

Armillaria is a primary fungal pathogen of tea, cause root rot of tea (*Camellia sinensis*) in Kenya. *Armillaria* species have been characterized by different methods based on morphology, somatic incompatibility and DNA profiles generated by PCR with RAPD, ISSR, RFLPs of the ITS and IGS region (Otieno et al., 2003).

Fusarium solani have been reported from Sri Lanka*. F. solani* isolate from infected tea showed 97% similarity in sequence to accession number HQ731048 which originated from Sri Lanka tea on the basis of identification done by using translation elongation factor (Freeman et al., 2012).

Some other tea diseases are red root rot caused by *Poria hypolateritia* Berk. Sacc**.,** brown root rot caused by *Phellinus maxius* Corner and Charcoal stump rot caused by *Ustulina zonata* (Chen and Chen, 1982) etc.

2.1.5. Identification of fungal pathogens

The correct identification of fungal phytopathogens is essential for virtually all aspects of plant pathology, from fundamental research on the biology of pathogens to the control of the diseases they cause. Conventional methods for identifying fungal plant pathogens rely on the interpretation of visual symptoms and/or the isolation, culturing and laboratory identification of the pathogen. The accuracy and reliability of these methods depend largely on the experience and

skill of the person making the diagnosis. Diagnosis requiring culturing can be time consuming and can be unfeasible when rapid results are required (McCartney et al., 2003). A newer method, which is increasingly being applied for diagnosis of plant pathogens by using polymerase chain reaction (PCR) amplification of nucleic acid sequences (Atkins and Clark, 2004). Several authors characterized and performed phylogenetic taxonomic studies of fungal phytopathogens from different sources by means of molecular techniques using ITS region (Huang et al., 2012; Jeewon et al., 2013). However, extremely little research has so far focused on PCR-based characterization of foliar fungal pathogens of tea.

Several authors characterized and performed phylogenetic taxonomic studies of fungal phytopathogens from different sources by means of molecular techniques using ITS region. Recently, Guo et al. (2014) isolated and characterized *C. gloeosporioides* a causal agent of tea leaf and twig blight in Gantang and Tangkou. Infected leaf tissues were collected from plants for determination of causal agent. Eleven isolates were recovered and all cultures produced white-to-gray fluffy aerial hyphae and were dark on the reverse of the plate. DNA based characterization and amplification of rDNA was done using the rDNA-ITS primer pair ITS4/ITS5, glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) primer pair GDF/GDR and beta-tubulin 2 gene (Tub2) primer pair Btub2Fd/ Btub4Rd. Sequence analysis of the 11 isolates showed that they were identical and revealed 100% similarity to the ITS sequence of strain P042 of *Colletotrichum gloeosporioides*, 100% identity to the GAPDH of isolate C07009 of *C. gloeosporioides* and 99% similarity to Tub2 of isolate 85 of *C. gloeosporioides* respectively. Based on overall data, the 11 isolates were identified as *C. gloeosporioides* (Penz.) Penz. & Sacc. To confirm pathogenicity, Koch's postulate was performed with each of the 11 isolates. All isolates confirmed as brown necrotic lesions appeared on leaves and twigs, while the control plants remained healthy.

2.1.6. Characteristics of fungal pathogens of tea

2.1.6.1. *Exobasidium vexans*

Exobasidium vexans Massee is an obligate parasitic Basidiomycete that occurs on the young leaves and shoots of the commercial tea bush which cause blister blight. *E. vexans* has no known alternative host and the life cycle is completed on tea plant itself (Subba Rao, 1946). It reproduces through basidiospores which are disseminated by wind. The spores are hyaline, elliptical and single-celled when immature and two-celled at maturity measuring 7-15.5 X 2.3-4.5 µm. The life-cycle of the fungus is completed in a short period of 11-28 days (Gadd and Loos, 1948; Agnihothrudu and Mouli, 1990).

Chakraborty et al. (2002) studied the biochemical changes of tea plants growing in Darjeeling hills exposed to blister blight infection caused by *E. vexans.* Biochemical levels of proteins, proline, phenols and enzyme activity such as polyphenol oxidase, peroxidase and phenyl alanine ammonia lysate were measured. It was observed that the phenyl alanine ammonia lysate level decline while polyphenol oxidase and peroxidase level increase in infected leaves. Some other significant changes were also observed such as proline content increased in blister infected leaves. At early stage of infection, the protein content was found to decline. The total phenols were higher in infected leaves in comparison to healthy leaves.

Nagao (2012) reported the effect of aqueous vitamin B on the colony formation and maintenance of *E. vexans*. He took three aqueous solution of biotin, thiamine and calcium pantothenate, for *in vitro* effect on the growth of *E. vexans* and observed that the germination process of basidiospores of *E. vexans* differed from those of the other *Exobasidium* species. Vitamin B5 and calcium pantothenate was only partially effective in generating the thick germ tubes and to induce the initial colony formation, whereas amendment of biotin and thiamine to the media did not induce visible colony growth.

Jeyaramraja et al. (2010) investigated that the certain physical barriers and chitinase enzyme of tea plants play an important role in resistance to blister

blight. Tea clone (SA-6) was resistance to blister due to higher amounts of epicuticular wax and increased thickness of cuticle/epidermal layer, functioning as physical barriers to hyphal penetration of *E. vexans*. After chitinase assay and western blotting study, authors confirmed that the constitutive level of chitinase expression was higher in the resistant clone SA-6.

2.1.6.2. *Colletotrichum* sp.

Genus *Colletotrichum* is a major foliar fungal pathogen of a wide variety of economically important woody and herbaceous plants. Different species of *Colletotrichum* are primarily described as causal agent of anthracnose diseases although other maladies are also reported such as red rot of sugar cane, coffee berry disease, crown rot of strawberry and banana and brown blotch of cowpea (Lenne 2002). *Colletotrichum camelliae* cause brown blight in tea leaf is known in Ceylon, Java and India and is more prone to attack on the young leaves (Petch, 1923; Singh et al., 2006). It has been reported from Anhui province of China, that *C. gloeosporioides* cause twig blight of tea (Guo et al., 2014).

Anthracnose disease symptoms include limited, often sunken necrotic lesions on leaves, stems, flowers and fruits, as well as crown and stem rots, seedling blight etc. (Waller et al., 2002; Agrios, 2005). Symptoms of several devastating diseases, *caused by different Colletotrichum* species have been reported from Africa. The pathogens seriously affect cereals including maize, sugar cane and sorghum along with berries of coffee. Based on perceived scientific and economic importance, the genus has recently been voted as the eighth most important group of plant pathogenic fungi in the world (Dean et al., 2012).

Freeman et al. (2000) isolated *Colletotrichum* sp. from almond, avocado and strawberry of Israel and from almond isolates (of the pink subpopulation) of United States. All isolates were characterized by various molecular methods and compared with morphological identification. Taxon-specific primer analysis grouped the avocado isolates within the species *C. gloeosporioides* and the U.S.

almond and Israeli strawberry isolates within the species *C. acutatum.* Similarity of the above populations to that of *C. gloeosporioides* of avocado was between 92.42 and 92.86%. DNA sequence analysis of the entire ITS region supported the phylogeny inferred from the ITS1 tree of 14 different *Colletotrichum* species.

Abang et al. (2002) studied taxonomic status of *Colletotrichum* isolates associated with anthracnose disease of yam and determined the relationship among them. Based on morphology, they identified four isolates as *C. gloeosporioides* which were confirmed by PCR-RFLP and sequence analysis of the internal transcribed spacer region of the ribosomal DNA (ITS1-5·8S-ITS2) were used. Sequence analysis of the ITS1 region and the entire ITS region were highly similar (97–100% nucleotide identity) to *C. gloeosporioides*. Lubbe et al. (2004) studied several distinct species of *Colletotrichum* sp. associated with proteaceous hosts growing in various parts of the world. They identified the species on the basis of morphology, sequence data of the internal transcribed spacer regions (ITS-1, ITS-2), the 5.8S gene and partial sequences of the ß-tubulin gene.

Photita et al. (2005) isolated and identified *Colletotrichum* species from herbaceous plants based on molecular analysis and sequences of the rDNA internal transcribed spacers (ITS1 and ITS2). Morphological and phylogenetic analysis of *Colletotrichum* pathogens and endophytes showed that endophytic isolates were most similar to *C. gloeosporioides*. However, no pathogenic isolates clustered with endophytic isolates. The correlation between morphological and molecular-based clustering demonstrated the genetic relationships among the isolates and species of *Colletotrichum* and indicated that ITS rDNA sequence data were potentially useful in taxonomic species determination.

Xie et al. (2010) studied on 31 isolates of *Colletotrichum* sp. of strawberry anthracnose disease from Zhejiang Province and Shanghai City of China. On the basis of morphological characteristics, phylogenetic and sequence analyses, they identified 11 isolates as *C. acutatum*, 10 isolates as *C. gloeosporioides* and 10 isolates as *C. fragariae*. They used species-specific polymerase chain reaction

(PCR) and enzyme digestion methods for further confirmation and identification of the *Colletotrichum* sp.

Kamle et al. (2013) isolated and identified *Colletotrichum gloeosporioides* from diseased mango samples showing anthracnose symptoms. Further identification was done by DNA based amplification of the internal transcribed spacer region of rDNA region 5.8S by using ITS primers. Amplicons showed sequence similarity of 100% with some *C. gloeosporioides* isolates. Park and Seo (2013) isolated *Colletotrichum gloeosporioides* from smoothlip cymbidium in Korea. In potato dextrose agar medium the colonies were olive to gray on the upper side and dark gray to black on the reverse side. Conidia were hyaline, cylindrical with both ends rounded, with size range of 13.7 to 19.2 x 4.0 to 6.1 µm. The identities of two representative isolates were confirmed by sequencing the internal transcribed spacer (ITS) regions and the large subunit rDNA. BLAST analysis of the sequences from each isolate against the GenBank database found 99% similarities to more than 30 accessions of *C. gloeosporioides.*

2.1.7. Antigenic relationship between host and pathogen

Common antigenic relationship among closely related organisms or even among more distantly related organisms is surprising (Alba et al., 1983; Eibel et al., 2005). Studies on both animal and plant hosts and their parasites and pathogens suggest that whenever an intimate continuing association of cells of host and pathogen occurs, partners of this association have a unique serological resemblance to one another involving one or more antigenic determinants. In plants, several studies have shown that the possibility of susceptibility is greater when antigenic similarity is greater. Thus the concept of common antigens between a plant and a pathogen is a notable feature in determining resistance or susceptibility. It is believed that the degree of compatibility and susceptibility of a plant cultivar to a pathogen is correlated to levels of common antigens present in both host and pathogen (Purkayastha and Banerjee, 1990; Chakraborty and

Saha, 1994; Ghosh and Purkayastha, 2003; Kratka et al., 2002; Musetti et al., 2005; Dasgupta et al., 2005).

The degree of compatibility and susceptibility of a plant cultivar to a pathogen is correlated to levels of common antigens present in both host and pathogen (Charudattan and DeVay, 1972; Alba et al., 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994; Ghosh and Purkayastha, 2003; Dasgupta et al., 2005). Immunodiffusion and immunoelectrophoresis can detect common antigenicity between susceptible varieties and the pathogen while ELISA can detect very low levels of common antigens in both host and pathogen even between moderately susceptible and resistant varieties (Alba and DeVay, 1985; Mohan, 1988; Chakraborty et al., 1995; Croft, 2002). Among the immuno-techniques, indirect ELISA is most sensitive and reliable in detecting the common antigens and it may be utilized to determine the pathogenicity of a fungus in different varieties of tea to a level of greater accuracy. This would be most useful in screening commercially cultivated tea varieties for susceptibility towards the fungal pathogens. Cloned cuttings of resistant varieties may be selected for planting in tea gardens which would thereby constitute a long-term disease control system.

Scala et al. (1994) observed the presence of cross reactive antigen (CRA) between pea and some fungal plant pathogens which showed different levels of specificity towards the host. Chakraborty et al. (1997) reported that the cross reactive antigens between the host and the pathogen were detected first by immunodiffusion. Polyclonal antisera were raised against the mycelia suspension of *Fusarium oxysporum* and the root antigen of the susceptible cultivar UPSM-19. They purified antiserum by ammonium sulfate precipitation and DEAE-Sephadex column chromatography. The immunoglobulin fractions were used for detection of cross-reactive antigens by enzyme-linked immunosorbent assay (ELISA). Results revealed that the antigens of susceptible cultivars showed higher absorbance values when tested against the purified anti *F. oxysporum* antiserum. Indirect staining of antibodies using fluorescein isothiocyanate indicated that in cross-

sections of roots of susceptible cultivar (UPSM-19) cross-reactive antigens were concentrated around xylem elements, endodermis and epidermal cells, while in the resistant variety, fluorescence was concentrated mainly around epidermal cells and distributed in the cortical tissues.

Chakraborty et al. (2002) reported that, pathogenicity of *Glomerella cingulata*, causal agent of brown blight disease, tested under identical conditions following detached leaf and cut shoot inoculation techniques against fourteen varieties of tea showed CP-1 and TV-26 as resistant and TV-18 and Teen Ali 17/1/54 as susceptible. The cross reactive antigen (CRA) was found among the susceptible varieties of tea and isolates of *G. cingulata*. Such antigens were not detected between resistant varieties of tea and isolates of *G. cingulata*. The polyclonal antisera were raised separately against antigen preparations from mycelia and cell wall of *G. cingulata*, mycelia of *Fusarium oxysporum* and leaf antigens of TV-18 and CP-1.

Dasgupta et al. (2005) detected common antigens between *Curvularia eragrostidis* and different tea varieties. Cross-reactive antigen (CRA) between the susceptible varieties (TV-11, 12 and 18) and *C. eragrostidis* were detected by immunodiffusion and immunoelectrophoresis. The presence of common antigens between *C. eragrostidis* and susceptible tea varieties was also evident by indirect ELISA values. Indirect ELISA could readily detect CRA in semi-purified preparation of antigen at concentration of 20µgml^{-1} with antiserum dilution of 1:125. The lower ELISA values indicate the disparity while higher ELISA values indicate similarity of antigens in cross reactions. Chakraborty et al. (2005) reported *Alternaria alternata* as a new foliar fungal pathogen of tea. A polyclonal antibody was raised against the virulent isolate of *A. alternata* and degrees of susceptibility to the pathogen against different varieties were tested. It showed the variable degrees of susceptibility to the pathogen apart from TV-28, UPASI-2 and UPASI-8 that were resistant. Tea varieties T-17, T-78 and TV-22 were found to be highly susceptible.

Schmechel et al. (2006) demonstrated that monoclonal antibodies (mAbs) and immunoassays have the potential to detect *Stachybotrys chartarum* species-specifically. They also described the isolate-dependent antigenic variability and cross-reactivity patterns of *S. chartarum* with other fungi commonly established in indoor environments and discussed the implications of biological and analytical variability on the development of immunometric environmental and serological monitoring assays for fungi. They also observed reactivity patterns with cross-reactive mAbs suggesting that, several fungi may share common antigens and that the majority of antigens are expressed by spores and mycelia. The observed cross-reactivity patterns need to be considered for accurate interpretations of environmental and serological analyses.

Chakraborty and Sharma (2007) reported that, the immunological methods proved to be valuable in screening of commercially cultivated tea varieties against *Exobasidium vexans*. They studied the pathogenicity of *Exobasidium vexans*, causal agent of blister blight of tea, against 30 commercially cultivated tea varieties by analysing the antigenic patterns of host and pathogen using immunological techniques. Antigen preparations from tea varieties, pathogen, nonpathogen and of nonhosts were compared by indirect enzyme-linked immunosorbent assay and dot-immunobinding assay using polyclonal antibodies raised against the pathogen, nonpathogen, susceptible and resistant tea varieties. Cross-reactive antigens were found among susceptible varieties and *E. vexans* isolates but not in resistant varieties, nonhosts or nonpathogen.

Mohan (1988) evaluated the susceptibility of strawberry plants to *Phytophthora fragariae* for detection of red core disease by enzyme-linked immunosorbent assay. Antiserum was raised against pooled mycelial suspensions from five isolates (designated Pf 1, Pf 2, Pf 3, Pf 10 and Pf 11) representing five physiologic races of *Phytophthora fragariae.* In enzyme-linked immunosorbent assay (ELISA), this antiserum detected homologous soluble antigens at protein concentrations 2 ng/ml. The cultivar Red Gauntlet, which is resistant to Pf 1, 2 and 3 but susceptible to Pf 10 and 11, reflected this differential response in

ELISA; the absorbance produced by extracts of plants infected with virulent isolates was significantly higher than that obtained with the corresponding extracts of plants inoculated with a virulent isolates.

Abou-Taleb et al. (2009) studied the role of common antigens in relation to potato resistance to late blight pathogen *Phytophthora infestans* by using indirect ELISA and dot blot immunoassay. The cross-reactivity was detected between antigens of potato cultivars (resistant and susceptible) and *Phytophthora infestans*. The outcome was indicated that, the higher reaction and more specific antigens were detected in the susceptible cultivars than in the resistant ones.

Saha et al. (2010) demonstrated that, the indirect ELISA method was most sensitive and reliable among the immunotechniques in detecting cross-reactive antigen (CRA) between susceptible eggplant varieties and *C. gloeosporioides*. The CRA shared by susceptible varieties and pathogen was also detected by immunodiffusion and immunoelectrophoresis. Such antigens could not be detected between the resistant varieties and the pathogen and also between a non-pathogen (*Alternaria porri*) and all the tested varieties. However, ELISA showed that low levels of common antigens were present between all combinations. They also observed the level of CRA was decrease with increasing resistance. The level of CRA was found to correlate to the pathogenicity of *C. gloeosporioides* in different eggplant varieties.

El-Komy et al. (2010) investigated the specificity and sensitivity of serological tools for the detection of *Phytophthora infestans* in infected tissues of susceptible and resistant potato cultivars and to study disease development among these cultivars. Several serological tools, enzyme-linked immunosorbent assay and dot blot immunoassay were performed using antiserum raised from soluble mycelial protein of *Phytophthora infestans*.

2.2. Materials and methods

2.2.1. Collection of samples

Samples of blister blight affected tea leaves were collected during May 2009 to February 2012 from various regions of Himalayan and sub-Himalayan region of West Bengal, which included the tea gardens of Darjeeling and Jalpaiguri districts (Fig. **2.1**). Desired tea leaves showing visible symptoms of blister blight affecting the region that is harvested, were plucked as short twigs from the apex of branches and immediately packed carefully in sterile, chilled polyethylene bag; stored in ice box and transferred to the Plant Biotechnology Laboratory, Department of Biotechnology, University of North Bengal within 12-18hr. Geographic Information System (GIS) locations of the places of sample collection and their respective codes are presented in table **2.1**.

Table: 2.1. Regions of collection of blister blight affected tea leaf samples.

Place of Sampling[a]	Code assigned to fungal isolates	Time of sampling	GIS location	
			Latitude	Longitude
Happy valley T.E.(Darjeeling)	DW-01, DW-9, DW-4, OB-1	August, 2011	27^0 2'N	88^0 14'E
Margaret Hope T.E. (Kurseong)	RBK-2, CR-5, WBRK-1, WBRK-2, TTRK-1, TTRK-3, TTRK-6, TTRK-10	July, 2010	26^0 53'N	88^0 16'E
Gaya ganga T.E. (Bagdogra)	CC19	January, 2012	26^0 38'N	88^0 19'E
Kamal pur T.E. (Bagdogra)	KW-1, KW-5, KW-19, KW-30	January, 2011	26^0 42' N	88^0 18'E
Singijhor T.E. (Bagdogra)	SR-5, SR-6, SR-7, SR-13	January, 2012	26^0 41'N	88^0 17'E

[a]T.E. = Tea estate

2.2.2. Isolation of fungal culture

For isolation of fungal pathogen, the sample packets were opened aseptically and the infected leaves were rinsed carefully in sterile distilled water. The infected portions were cut into small pieces and surface-sterilized with 0.1% $HgCl_2$ for 1 min. The pieces were again rinsed thoroughly in sterile distilled water and placed on potato dextrose agar (PDA) medium in petriplates (9cm diameter). The plates were incubated for 4-5 days at 28°C. Preliminary isolations were done in PDA supplemented with the antibacterial streptomycin (Stevens, 1981). Isolated fungal colonies grown in the plates were picked up by inoculating needle and were placed in sterile PDA slants. The cultures were observed under microscope and pure cultures were sub cultured for further use.

2.2.3. Maintenance of stock cultures

Freshly prepared sterile PDA slants were used for the maintenance of the fungal cultures. Pathogens grown on sterile PDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 4°C) and at room temperature. At the interval of 15 days subculture was done for preparation of inoculums for different experiments.

2.2.4. Plant materials

The tea plant varieties were selected on the basis of susceptibility to fungus (Chakraborty and Saha, 1994; Chakraborty et al., 1995; Dasgupta et al., 2005). Additionally the most cultivated varieties were given priority. Thus eight different tea varieties were selected for immunological detection of sensitivity/resistivity towards the isolated pathogen of tea. These varieties (TV-3, TV-9, TV-18, TV-22, TV-25, TV-26, TV-29 and TV-30) were collected from different nurseries of tea gardens of the Darjeeling district. They were planted in earthenware pots (one plant per pot of 25 cm diameter) and maintained in the open in the experimental garden of Department of Botany, University of North Bengal (Dasgupta et al., 2005).

2.2.5. Pathogenicity test

2.2.5.1. Inoculum preparation

For inoculum preparation, the fungal pathogen was grown in PDA plate for 10-12 days at 28ºC for adequate sporulation. Spore suspension was prepared in sterile distilled water; which was added aseptically to the fungal cultures. The surface of the mycelial mat was brushed gently with inoculation needle to loosen the spores. The resulting suspension was filtered through four layers of muslin cloth to remove mycelial fragments. The concentration of spores in the filtrate was measured using haemocytometer and the final concentration was adjusted to 1 x 10^6 spores ml^{-1} (Saha et al., 2005a).

2.2.5.2. Assessment of Pathogenicity

Pathogenicity test was done by artificial inoculation of detached leave of tea with test pathogens following the detached leaf inoculation technique proposed by Dhingra and Sinclair (1995). Initially, pathogenicity of all isolates was tested in TV-18 and TV-30 varieties. The fungi which were detected as pathogenic were further screened against other six varieties (TV-3, TV-9, TV-22, TV-25, TV-26 and TV-29).

To perform the experiment, fresh young fully expanded and detached host leaves (5-7 cm long) were detached from third or fourth node of mature tea bushes of TV-18 and TV-30 varieties, cleaned thoroughly and placed on blotting paper moistened with sterile distilled water. The blotting paper was laid in trays and covered with a glass lid to form humid chambers. For inoculation, two wounds (light scratch of 5 mm length) were made with a sterile sharp needle on the adaxial surface of the leaves at either side of the midrib. The leaves were inoculated by placing 50 µl of the spore suspension on the wounds following the inoculation technique. Ten leaves were used in a set and the whole experiment was repeated thrice. A set of 10 leaves which received sterile distilled water instead of fungal inoculum was considered as uninoculated control. Trays were kept at 28^{o}C in a growth chamber with 12 h photoperiod. Each tray was covered

with a glass lid and sealed with vacuum grease in order to maintain the required moistures inside the trays during incubation. The frequency of infected leaves and diameter of lesion (if any) was recorded. For each sets of experiment five leaves were taken.

2.2.5.3. Verification of Koch's postulates

Pathogenicity of the isolated pathogen of tea was confirmed by verification of Koch's postulates. Pathogenicity was tested by whole plant inoculation method of Dickens and Cook (1989). Well established young tea plants (3 month old) of TV-18 and TV-30 varieties grown in pots were inoculated by spraying fungal suspension of test pathogen. For preparing fungal inoculum, ten day old sporulated culture of the test pathogen CR5 (grown in 100ml PDB in 250 ml flasks) was harvested and the mycelial mat was collected by filtration through sterile muslin cloth. The mycelium (50g) was mixed with sterile distilled water (200 ml). The mixture was blended properly to obtain fragmented mycelial mat. In control sets, plants were sprayed by sterile distilled water. The plants were kept for 48 hours in perforated transparent polythene chambers, previously mist-sprayed with sterile distilled water for maintaining high humidity. Subsequently the plants were removed from the chamber and grown in green house with normal soil surface watering under natural light and temperature. Humidity was maintained by spraying sterile distilled water at intervals. The experiment was repeated thrice taking 10 plants in each replication. Observation for disease development was done after 4, 8 and 12 days of inoculation. After 14 days of incubation and after disease symptoms appeared on the leaves, the pathogen was re-isolated from infected leaves. During re-isolation, infected portions of the leaves were cut into small pieces (1-2cm), washed thoroughly with sterile distilled water, surface sterilized with 0.1% $HgCl_2$ for 1 minute, washed three times with sterile distilled water and finally transferred aseptically into sterile PDA slants. The inoculated slants were incubated at 28^{o}C and were observed till sporulation. Sporulated cultures were used for microscopic studies and preliminary identification of pathogen after

staining with lactophenol in cotton-blue as stated earlier. The identity of the organism was confirmed after microscopic observation; comparing it with the stock culture. If an organism was consistently re-isolated then it was treated as a pathogen.

2.2.6. Characterization of the isolated fungus

2.2.6.1. Morphological characterization and microscopy

The morphology of the isolated fungal pathogen was studied by first inoculating the fungus on PDA plates and incubating for 3-5 days for the culture to develop. The plates were observed for formation of fungal growth, color of mycelial mat and growth pattern. For microscopic observations, mycelia were taken from pure culture on clean and grease free microscopic slides and stained using cotton-blue in lactophenol. The slides were mounted with cover glass, sealed and observed under compound microscope at 40x and 100x (oil immersion) (Olympus, India). Length and breadth of spores and breadth of mycelia were measured by calibrated ocular micrometer with respect to the stage micrometer of the microscope. The details of the morphology of the fungus were noted.

To study the shape and size of spores and hyphae of the biotrophic fungus, a bit of hyphae and spores were scrapped out by a sterile needle from a portion of blister formed on the underside of a freshly plucked affected leaf and placed on a sterile slide. A drop of lacto-phenol in cotton blue was added to it and the clump of hyphae was separated carefully using sterile needles. Spore suspension was prepared directly from natural blisters on tea leaves. The pieces of blister region were taken in sterile distilled water and vortexed for 1 min. The resulting mixture was filtered through 2 layers of sterile cheese cloth to obtain the spore suspension. A drop of spore suspension was placed on a clean microscopic slide and stained for 1-2 min with lacto-phenol in cotton blue. Glass cover slips were placed over the fungal preparations and observed under microscope**.** The shape of spores was noted and size was measured with standard ocular micrometer as stated above.

2.2.6.2. Phylogenetic characterization

Phylogenetic characterization of the pathogenic fungal isolates was carried out by partial sequencing of the 18S rRNA gene. The genomic DNA was isolated either from cultured pathogenic fungal mycelia (for culturable non biotroph) or from the tea leaves (for biotroph) and used as template for amplification of the target gene. The sequences obtained were subjected to Blast searches for finding similarity with other sequences of related species and the results were analysed for identification of the fungal strains.

2.2.6.2.1. Isolation of genomic DNA from infected leaves by CTAB method

DNA was isolated from leaves by the method of Doyle and Doyle (1987) with some modifications. The collected fresh samples of blister affected leaves were washed thoroughly with sterile distilled water and the infected parts were chopped into small pieces and submerged into ethanol for 30 minutes. The leaf tissue samples were then removed from ethanol and dried on sterile petridish in air. Samples were stored frozen at -20^{o}C for 2 hours or until DNA isolation. Frozen leaf tissues (1gm) were taken in a chilled mortar and pestle and ground to homogenize using 2% CTAB DNA extraction buffer (5ml). The homogenized suspension (0.8ml) was taken in 1.5ml eppendorff tubes and incubated for 1 hour at 65^{o}C water bath, occasionally mixing by gentle swirling. Following incubation, 0.6 volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tubes and mixed by inversion for 15 min. Solutions were centrifuged at 10,000 rpm for 15 minutes and supernatant of each sample were transferred into fresh tubes. Equal volume of chilled isopropanol (-20^{o}C) was added to the tubes to precipitate the DNA and mixed gently by inverting about five times. The precipitated DNA was centrifuged at 2,000 rpm for 2 minutes at 4^{o}C. The supernatant was discarded and the DNA pellet was retained. The pellet was washed with 70% ice cold ethanol (40µl) and allowed to stand at room temperature for 20 minutes. Finally the sample was centrifuged at 7,000 rpm for 5 minutes at 4^{o}C. The pelleted DNA was dried overnight at room temperature and finally dissolved in 40µl of 1X TE buffer.

2.2.6.2.2. Isolation of genomic DNA from fungus by CTAB method

DNA from fungal cultures was isolated following a modified method (Joshi et al., 2009) of Doyle and Doyle (1987). The fungus was grown in 150 ml PDB taken in 500 ml conical flasks for 3-5 days. The fungal mycelium was harvested by filtering through sterile cheese cloth and stored overnight at -20^{o}C. The frozen mycelium (1g) was removed from refrigerator and immediately crushed in a chilled mortar and pestle and homogenized in 5 ml CTAB extraction buffer. The homogenate was taken in 1.5ml eppendorff tubes and incubated for 1 hour at 65^{o}C water bath with occasional mixing. Then, 0.6 volume of chloroform: isoamyl alcohol (24:1) was added, mixed for 15 min by inversion and centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to fresh tubes to which equal volume of chilled isopropanol (-20^{o}C) was added and mixed gently. The DNA precipitate was centrifuged at 2,000 rpm for 2 minutes at 4^{o}C following which the pelleted DNA was washed with 70% ice cold ethanol (40µl) by centrifugation at 7,000 rpm for 5 minutes at 4^{o}C. The DNA pellet was dried overnight at room temperature and finally dissolved in 40µl of 1X TE buffer.

2.2.7. RNAse treatment

The RNA present in the extracted total genomic DNA was removed by RNase treatment. The genomic DNA solution (100µl) in 1X TE buffer was incubated with RNAse (60µg) at 37^{o}C for 30 minutes. Following incubation, DNA was re-extracted with equal volume of saturated PCI (Phenol: chloroform: isoamylalcohol 25:24:1) which was added to the DNA solution and mixed by swirling for 5 min. After centrifugation of the mixture at 10,000 rpm for 5 min, supernatant was collected in a fresh tube and DNA was precipitated with chilled ethanol. The DNA was washed twice with ethanol, dried and resuspended in 30µl TE buffer (Maniatis et al., 1982). The purified DNA sample was stored at -20^{o}C for future use.

2.2.8. Quantification of DNA

Purity of DNA was estimated from the OD_{260}/OD_{280} ratio. For this, the optical density was recorded at wavelengths of 260 nm and 280 nm. The reading at 280 nm value showed the amount of protein in the sample. The reading at 260 nm gives the concentration of nucleic acid in the sample. Pure preparation of DNA has OD_{260}/OD_{280} value in the range of 1.8 to 2.0. The standard value of 1 OD at 260 nm for double stranded DNA corresponds to 50ng/µl of dsDNA. For quantification of isolated DNA, the DNA sample (1µl) was diluted in 49 µl TE buffer (dilution factor = 50) and OD was recorded in a dual beam Varian Cary 50 Bio UV-Visible spectrophotometer (Varian, Australia).

2.2.9. Gel electrophoresis

The quality and quantity of isolated genomic DNA was checked on agarose gel by agarose gel electrophoresis conducted in a submarine gel electrophoresis system (Bangalore genei (India) Pvt. Ltd., India). Agarose (0.8%) was suspended in 1X TAE buffer, melted in water bath till clear solution was obtained. The solution was allowed to cool to about 50-60^{o}C, ethidium bromide (0.5µg/ml) was added, mixed properly and mixture was poured into the gel casting tray. After solidification, the gel with tray was completely submersed in tank containing 1X TAE running buffer. DNA sample (6µl) were mixed with 2µl gel loading buffer and loaded onto wells. Electrophoresis was run at 55-58 volt for 1 hour and viewed under UV transilluminator (Bangalore Genei, India). Presence of bright fluorescent orange bands indicated the presence of DNA.

2.2.10. Amplification of 18S rRNA gene using Polymerase Chain Reaction (PCR)

PCR amplification of the fungal 18S rRNA gene was performed using PCR Amplification Kit (Banglore Genei, India). PCR mixture included 2.5mM $dNTP_{mix,}$ 15mM taq buffer with $MgCl_2$, 0.2µM of each primer ITS1 and ITS4 (White et al., 1990) and 1.5U Taq. DNA polymerase. Taq. DNA polymerase was added after adding all the ingredients of the mixture. PCR was performed in 25µl reaction

volume containing 2µl of ten times diluted genomic DNA using the conditions: initial denaturation at 94^{o}C for 5 min followed by 30 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 53.5^{o}C for 30 sec, extension at 72^{o}C for 30 sec and a final extension at 72^{o}C for 5 minutes on a thermal cycler (Applied Biosystems GeneAmp PCR 2400).

2.2.11. Detection of PCR amplicon in agarose gel

The PCR amplicon was resolved on 1% agarose gel containing ethidium bromide following methods described earlier (section **2.2.9**). The molecular weight of PCR products were measured by using molecular ladder (100bp) runs parallel to samples. The resolved amplicons were observed under UV transilluminator and photographed. Finally the molecular weights of the amplicons were noted.

Table: 2.2. List of primers used in this study

PCR targets	Primers	Primer sequences
rDNA/ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3,
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'
Recombinant DNA	SP6	5'-ATTTAGGTGACACTATAG-3'
	T7	5'-TAATACGACTCACTATATAGG-3'

2.2.12. Restriction digestion

The amplified 18S rRNA gene were analysed by *Cfo*I restriction pattern. Reaction was conducted in a small polypropylene tube where 20 µl of the total reaction mixture contained 10X RE buffer, 1 µg DNA (PCR product), 0.2µl acetylated BSA and 5U of the *Cfo*I enzyme (Promega Corporation, Madison, USA). The mixture was incubated overnight at 37^{o}C. The digested DNA was precipitated using ethanol (40µl) and centrifuged at 10,000 rpm for 5 minutes. Pellets were re-suspended in TE buffer (10µl). The sizes of the restriction fragments were evaluated by resolving the digest on 2.5% agarose gel with 100 kb markers run side-by-side on

an agarose gel containing ethidium bromide. Electrophoresis was performed using 1X TAE running buffer at 58V for 60 min and visualized under UV light in a transilluminator.

2.2.13. Cloning of PCR products

The successfully amplified 18S rRNA gene portions of pathogenic fungal strains were cloned before proceeding for sequencing. The pGEM T-easy Vector System II, purchased from Promega Corporation, Madison, USA, was used following manufacturer's instructions. The PCR products were cloned in the pGEM-T easy vector without purification of PCR products. For vector ligation, the reaction mixture was prepared by adding 5µl of 2X rapid ligation buffer, 1 µl pGEM T-easy vector (50ng), 1.5µl PCR products (200-300ng) and 1µl T4 DNA ligase (3U/µl). A final volume of 10µl was adjusted with deionized water. Ligation reaction was thoroughly mixed by vortexing and incubated overnight at 4^{o}C.

2.2.13.1. Preparation of competent cells

A single colony of *E. coli* JM109 from a plate freshly grown for 16-18 hours at 37^{o}C was picked and transferred into 10ml of LB broth in a 100ml flask. The culture was incubated for overnight at 37^{o}C with shaking at 150 rpm. The overnight grown cultured in LB broth was aseptically transferred (100µl) in 10ml of fresh LB broth medium and grown at 37^{o}C in shaking for 2 to 4 hours (until OD=0.4 to 0.6 at 600 nm). The cells were transferred aseptically to sterile microcentrifuge tubes (1.5ml) and harvested by centrifugation at 6,000 rpm for 7 min at 4^{o}C. The medium was decanted; pellet was re-suspended in 1ml ice cold solution-I (80mM $MgCl_2$ and 20mM $CaCl_2$) and mixed gently. The cells were recovered by centrifugation at 4,000 rpm for 7 minutes at 4^{o}C. The supernatant was discarded and the cell pellet was re-suspended in 1ml solution-II (100mM $CaCl_2$). The contents were gently mixed and stored in ice for 45 minutes. The tubes were again centrifuged at 4,000 rpm for 7 min; the supernatant was discarded and the

retained pellet was re-suspended in 500 µl Solution-II. The resulting cells were competent cells and were further used in the study.

2.2.13.2. Transformation of cloned DNA

Overnight stored competent *E. coli* JM109 cells at 4^{o}C were used for transformation of insert ligated vector DNA. To 100µl competent cells taken in fresh microcentrifuge tube, 10µl prepared ligation mixture was added and mixed by gentle swirling. The tubes were stored on ice for 45 minutes. The cells were then incubated in a preheated circulating water bath at 42^{o}C for exactly 90 seconds and rapidly transferred to ice bath where it was held for 10 minutes. Next, an aliquot of 300µl LB medium was added to the tube and incubated for 1-2 hours in incubator shaker at 37^{o}C to allow the bacteria to recover and to express antibiotic resistance marker encoded by the plasmid.

2.2.13.3. Blue white screening

An aliquot of 100 µl of transformed cells were pipetted out from the microcentrifuge tube and spread on pre-warmed (at 37^{o}C) LB plate containing ampicillin (20 µg/ml), IPTG (0.5 mM) and X-Gal (80 µg/ml) with the help of plate master (Hi Media Laboratories, India). All plates were kept inside the laminar air flow for 10 min for absorbing the media, sealed with parafilm and incubated in an inverted position at 37^{o}C for overnight. The plates were then observed for formation of blue and/or white colonies. The white coloured recombinants were selected and used for sequencing of the cloned insert.

2.2.13.4. Identification of target DNA fragment in recombinant cells

Before sequencing of the cloned insert, the presence of the target DNA was confirmed by PCR using vector specific primers SP6 and T7. White colonies were selected and picked with plastic toothpicks and lysed by boiling in distilled water for 15 minutes. After that hold at room temperature for cooling then centrifuged at 7,000 rpm for 5 min. 2µl for each lysate (supernatant) was used as template in PCR reaction using the gene specific primers (either ITS1 & ITS4/ SP6 & T7)(table

2.2). The reaction set-up was as described above in PCR. The PCR products were electrophoresis on a 1.5% agarose gel with ethidium bromide stained as described above. The clones yielding different sized PCR products were chosen for sequencing service from Bangalore, India.

2.2.14. Sequencing of cloned PCR products

After obtaining the positive clones, they were sequenced at Bangalore Genei Sequencing Services, India and Xcerlis Labs, Gujarat, India. The sequences of the amplicons are listed in Appendix-D. The partial 18S rRNA gene sequences were submitted to NCBI GenBank. The fungal strains were identified by similarity searches of the sequences using the BLAST function of GenBank (Altschul et al., 1997; Tamura et al., 2007).

2.2.15. Phylogenetic analysis

The partial 18S rDNA sequences of the pathogenic fungal strains were compared with available 18S rDNA sequences of other similar strains in GenBank databases using the BLAST search facility at the National Center for Biotechnology Information (NCBI). The phylogenetic trees were constructed with the neighbor-joining method by using MEGA 4.0 (Tamura et al., 2007). Confidence in the tree topology was determined by bootstrap analysis using 1000 re-samplings of the sequences (Felsenstein, 1985).

2.2.16. Preparation of Antigen

2.2.16.1 Antigen from fungal mycelia

The antigen was prepared as total protein extracted from mycelia of *C. gloeosporioides* isolate CR5 following the method described by Dasgupta et al. (2005) and Chakraborty and Saha (1994). Mycelial discs (4 mm diameter) from 5 days old PDA culture plates were transferred to 10 conical flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28^{o}C. The fungal mycelia were harvested after 15 days, filtered through muslin cloth, washed with 0.2% NaCl and rewashed twice with sterile distilled water. The

mycelial mat was stored for an hour at -20^{o}C. Frozen mycelia (20g fresh weight) was homogenized with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% sodium chloride in a mortar and pestle with sea sand and kept overnight at 4^{o}C. The homogenate was centrifuged at 4^{o}C for 30 min at 10,000 rpm. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and again kept overnight at 4^{o}C. Next, the mixtures were centrifuged at 4^{o}C for 30 minutes at 10,000 rpm. The supernatants were discarded and precipitates were dissolved in cold 5ml 0.05 M sodium phosphate buffer (pH 7.4). The preparations were dialyzed for 24 hours at 4^{o}C against 0.005 M sodium phosphate buffer (pH 7.4) with 10 changes. After dialysis, the soluble protein was concentrated over polyethylene glycol 6000. The protein content was determined following the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Concentration was adjusted to 10mg/ml in 0.05 M phosphate buffer and stored at -20^{o}C as antigen until further use.

2.2.16.2 Preparation of antigen from tea leaf

The protein extraction procedure of Alba and DeVay (1985) and Chakraborty and Saha (1994) were followed in order to extract leaf antigens from young tea leaves. Fresh young leaves of the required varieties of tea were collected from green house of experimental garden of Department of Botany, University of North Bengal. The collected leaves were washed thoroughly with cold water and kept for 2 hours at -20^{o}C. The cold leaves (20 g fresh weight in each case) were ground in prechilled mortar at 4^{o}C with 20 g of insoluble polyvinyl pyrrollidone (PVP). The leaf paste was suspended in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride and 0.02 M ascorbic acid. The leaf slurry was strained through muslin cloth and centrifuged at 4^{o}C for 30 minutes at 10,000 rpm. The supernatants were collected and ammonium sulphate was added at 4^{o}C to 100% saturation under constant stirring, kept overnight 4^{o}C and finally centrifuged at 4^{o}C for 30 minutes at 10,000 rpm. The precipitate obtained was dissolved in cold 0.05 M sodium phosphate buffer (pH 7.0) and was dialysed

against 0.005 M sodium phosphate buffer (pH 7.0) for 24 hours at 4^{o}C with 10 changes. After dialysis, the preparation was centrifuged at 4^{o}C for 30 minutes at 10,000 rpm and supernatant was stored at -20^{o}C until required.

2.2.17. Immunisation

Normal serum was collected from rabbit before immunisation. Antiserum against antigen of pathogen was raised in white rabbits (New Zealand), which were immunized subcutaneously with 2-5mg of protein extract (0.5ml) of fungal pathogenic strain *C. gloeosporioides* (CR5) emulsified with an equal volume of Freund's complete adjuvant. For the first injection, the isolated protein was emulsified with an equal volume of Freund´s complete adjuvant (Difco lab, USA) and subsequent booster doses were repeated at 7days interval with Freund's incomplete adjuvant for seven consecutive weeks. The final protein concentration was 2.5 mg/ml in the emulsion. On the fourth day after the last injection, blood was collected from rabbit by puncturing the marginal ear vein in a sterile glass graduated tube. Blood samples were kept undisturbed for an hour at 37^{o}C for clotting. Finally, antiserum was clarified and carefully separated by micropipette, distributed in sterile cryo vials and stored at -20^{o}C until required. The normal serum from unimmunized rabbit was obtained after similar processing and stored at -20^{o}C in sterile cryo vials.

2.2.18. Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Indirect ELISA was performed by following the methods of Koenig and Paul (1982) and Talbot (2001). At first, antigens were diluted with coating buffer (0.1 M carbonate buffer, pH 9.6) and 100µl of each diluted antigen was placed on the wells of a flat bottomed micro titer ELISA plate (Tarsons) except one well which was considered as blank. The plate was incubated overnight at 4^{o}C in refrigerator. After incubation, the plate was taken out and each well was flooded with 0.15 M phosphate buffer saline (PBS)-Tween (pH 7.2) and washed thoroughly four to five times. After washing the plate was dried in air. Following this, 100µl of PBS-BSA

(0.15 M PBS containing 1% BSA) was added to each well to coat all the unbound sites. The plate was incubated for 2 hours at room temperature. After incubation, the plate was again washed with PBS-Tween and air-dried. After this, 100µl of diluted antisera (diluted with PBS-Tween) were added to each well except the blank and the control wells where normal sera was added (serially diluted with PBS-Tween containing 0.5% BSA). The plate was incubated overnight at 4^{o}C. In the next day, thorough washing of the plate was done with PBS-Tween. After washing and drying, 100µl (1:10,000) goat-anti rabbit IgG-horse radish peroxidase conjugate was added to each well except the blank and the plate was incubated for 2 hours at 30^{o}C. After incubation, the plate was again washed with PBS-Tween and shaken dry. Then 100µl (1:20) of tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. After addition of substrate, a blue colour was produced due to the reaction between the enzyme and the substrate. Finally, the reaction was terminated after 30 min by adding 100µl of 1(N) H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm.

2.3. Results

2.3.1. Isolation of fungi and verification of Koch's postulates

Altogether six fungal cultures (CR5, CC19, TTRK-1, TTRK-3, TTRK-6 and TTRK-10) were obtained in PDA. Each of these was tested for their pathogenicity in detached leaves of tea. Of these CR5 was found to induce disease symptoms. The symptoms appeared as brownish spots which gradually increased in size and turned dark. CR5 was further used for verification of Koch's postulates.

All inoculated plants of TV-18 and TV-30 variety showed typical symptoms of brown blight after 8 to 12 days which grew severe by 14^{th} day. TV-18 was found to be less susceptible as it produced smaller lesions. The isolated fungal culture was later identified as *Colletotrichum gloeosporioides* strain CR5. This re-isolation of the pathogen from infected plants confirmed it as a pathogen of tea and thereby the Koch's postulations were verified.

2.3.2. Morphological characters

The morphology of the pathogenic isolate was observed after growing in PDA. The mycelia were initially white in colour, which gradually turned pale yellow and further darker to gray (Fig. 2.2A,B,C). Huge masses of pinkish acervuli were produced. Mycelia and conidia of the fungus were light colored. The length and breadth of the mature conidia were 13-16 µm and 4-6 µm respectively. The mature conidia were light, one-celled and hyphae were septate, the diameter of the mature hyphae was between 3-5 µm (Fig. 2.2D).

The spores of the biotroph which was collected directly from the blisters (Fig. 2.3A & 2.3B) of diseased leaves were found to be hyaline, elliptical and single-celled or two-celled measuring 9-12 µm X 3-5 µm (Fig. 2.3C).

2.3.3. Phylogenetic characterization

The extracted and purified (RNase treated) DNA run in agarose gels from both plant and fungal sources produced intense fluorescent bands when viewed in a UV-transilluminator (Fig. 2.4). Amplification of the 18S rRNA gene followed by gel electrophoresis of the PCR product resulted in an amplicon of approximate band

size 600bp from the white blister samples and two close bands of similar size (600 and 650 bp) from red blister samples (Fig. 2.5). The PCR product obtained using fungal DNA as template also produced single band of 600 kb size (Fig. 2.5).

Cloning of the PCR amplification product in pGEM T-easy vector and subsequent transformation of *E. coli* JM109 cells led to the successful identification of transformed cells via blue white screening (Fig. 2.6). The transformation efficiency was recorded as 64%. The recombinant cells appeared as white colonies which were selected for confirming the presence of the target insert. The PCR using SP6 and T7 primers showed variation in the size of cloned insert among the blister samples (Fig. 2.7).

2.3.4. Restriction digestion of the cloned amplicons

Restriction digestion of the cloned amplicons was performed by using C*fo*I restriction enzyme. Results of the digestions were observed in agarose gel. On the basis of the different band patterns on the gel, differences among the clones were evident. Fourteen clones out of seventy two clones tested were found to be distinct on the basis of differences in their restriction digestion patterns (Fig. 2.8). All the thirteen samples were sent for sequencing.

2.3.5. Identification of the fungal isolates

The partial 18S rRNA gene sequences were obtained from the sequencing service provider. BLAST searches with these sequences revealed the presence of a number of fungi in the blister affected leaf samples. The identity of each fungus is listed in table 2.3. The fungal cultures that were isolated in PDA were also identified through BLAST searches and all identified sequences were submitted to NCBI GenBank and accession numbers were provided (Table 2.4).

2.3.6. Phylogenetic analysis of the fungal isolates

All 18S rDNA gene sequences obtained from the sequencing service provider was deposited in the NCBI GenBank and accession numbers were provided (Table 2.3). Alignment of the obtained 18S rDNA sequences with that of the strains from the

GenBank database resulted in 99 to 100% similarity matches for *C. gloeosporioides* While, in case of *Exobasidium* sp. 85-86% similarity matches from GenBank data bases. The phylogenetic relationship among the *C. gloeosporioides* and *Exobasidium sp.* isolates were established based on their partial 18S rDNA gene sequences. The phylogenetic tree was constructed based on the percent difference in genetic relationships between the allied strains in the NCBI database. Phylogenetic analysis was conducted using MEGA version 4.0. The results of analysis expressing the genetic relations of the *Exobasidium sp.* strains are represented in fig 2.9.

Table: 2.3. A list of fungal cultures isolated from infected tea leaves

Place of isolation[a]	Code	No. of clones selected	Identity	%Similarity Dated: 23.09.2013
Happy valley Tea Estate (Darjeeling)	DW-1	4	*Exobasidium sp.*	86
	DW-4		*Colletotrichum gloeosporioides*	100
	DW-9		*Colletotrichum gloeosporioides*	100
	OB-1		*Colletotrichum gloeosporioides*	100
Singijhor T.E. (Bagdogra)	SR-5	4	Uncultured fungus	99
	SR-6		*Exobasidium* sp.	85
	SR-7		*Exobasidium sp.*	85
	SR-13		*Sporobolomyces bannaensis*	99
Kamalpur T.E. (Bagdogra)	KW-1	3	Uncultured fungus	95
	KW-19		*Sporobolomyces* sp.	90
	KW-30		*Exobasidium sp.*	86
Gayaganga T.E.	CC19	1	*Colletotrichum gloeosporioides*	99
Margaret Hope T.E. (Kurseong)	RBK-2	7	Uncultured fungus	83
	CR-5		*Colletotrichum gloeosporioides*	100
	WBRK-1		Uncultured fungus	92
	TTRK-1		*Colletotrichum gloeosporioides*	99
	TTRK-3		*Neofusicoccum parvum*	100
	TTRK-6		*Neofusicoccum parvum*	99
	TTRK-10		*Fusarium solani*	99

[a]T.E. = Tea estate

Table: 2.4. A list of fungal isolates from infected tea leaves and their accession number

Fungal culture	codes	Accession number
Exobasidium sp.	DW-1	JX560484
Exobasidium sp.	KW-30	KC493154
Exobasidium sp.	SR-7	KJ400966
Colletotrichum gloeosporioides	OB-1	KC493155
Colletotrichum gloeosporioides	DW-4	KF837644
Colletotrichum gloeosporioides	DW-9	KF836745
Colletotrichum gloeosporioides	CR5	KC493156
Colletotrichum gloeosporioides	CC-19	KF836743
Fusarium solani	TTRK-10	KJ400973
Uncultured fungal endophyte	KW-1	KJ400971
Uncultured fungal endophyte	SR-5	KJ400968
Uncultured fungal endophyte	WBRK-1	KJ400969
Uncultured fungal endophyte	RBK-2	KJ400970
Sporobolomyces bannaensis	SR-13	KJ400967
Sporobolomyces sp.	KW-19	KJ400972
Neofusicoccum parvum	TTRK-3	KJ400973

2.3.7. Pathogenicity of the fungal isolate

The pathogenicity of *Colletotrichum gloeosporioides* was initially tested following the detached leaf technique on eight different tea varieties. The varieties TV-30 and TV-18 were considered highly susceptible after 72 hrs inoculation. Out of the eight tested varieties, TV-30 and TV-18 were found to be more susceptible

varieties while TV-22, TV-26, TV-29 and TV-3 were the moderately susceptibility and, TV-25 and TV-9 resistant. Brownish spots were produced that progressively turned black in centre surrounded by a yellowish brown region. A comparative result (figure 2.10) showed that TV-30 and TV-18 were highly susceptible whereas TV-25 was resistant.

2.3.8. Evaluation of susceptible varieties of tea against *Colletotrichum gloeosporioides* by Indirect ELISA

The leaf antigens of 8 tea varieties included in this study and antiserum raised against mycelial antigen of the fungal pathogen *C. gloeosporioides* were used to perform indirect ELISA. Raised antiserum against mycelial protein antigen was diluted 1:100 with 0.05 M phosphate buffer (pH 7.4). The diluted antiserum was tested against antigens at a concentration of 20µgml^{-1} which was prepared from different varieties of tea leaves. The results summarized in figure 2.11 revealed that cross reactive antigens were successfully detected between compatible antigen-antibody interaction that is, between pathogen and susceptible tea varieties. Susceptible varieties (TV-18 and TV-30) showed higher absorbance whereas the resistant varieties showed lower absorbance values. Thus TV-18 which was found to be most susceptible in pathogenicity tests showed a high absorbance at 1.8 while the resistant variety TV-25 recorded an absorbance of 0.56. This clearly indicated that cross-reactivity was higher between pathogen and susceptible variety than between pathogen and resistant variety. Results obtained from all the combinations showed that the absorbance values of normal serum control were lower than the corresponding test values.

2.4. Discussion

Almost all aspects of plant pathology require the correct identification of fungal phytopathogens which is essential not only for fundamental research on the biology of pathogens but also vital to the control of the diseases they cause. The molecular methods such as polymerase chain reaction are routinely used to detect

and identify plant pathogens. Previous studies on the use of rDNA gene sequencing as DNA barcode for species designation within the genus *Pestalotiopsis* have been reported (Gehlot et al., 2012). However, little research has focused on molecular-based characterization of other foliar fungal pathogens of tea.

Traditional methods for identifying plant pathogenic fungi include interpretation of visual symptoms, isolation, culturing for detecting and enumerating the fungal spores. Accuracy by this method requires skilled and highly specialized expertise. But the processes are time-consuming, laborious and not suitable when rapid results are required (McCartney et al., 2003). However, cultural techniques are inappropriate for detection of slow growing or non-culturable fungi *in vitro* (Lacap et al., 2003). The accurate identification of fungal phytopathogens is essential for virtually most of aspects of plant pathology from fundamental research on the biology of pathogens to the control of the diseases they cause.

Studies on fungal identification based on DNA sequences have often involved the Internal Transcribed Spacer (ITS) region of ribosomal DNA, which is located between the 18S and 28S subunit genes and repeated numerous times (White et al., 1990). Hirata and Takamatsu (1996) concluded that while the rDNA ITS region is conserved, it is sufficiently variable enough to facilitate phylogenetic studies of closely related species. Several recent phylogenetic studies have led to sequencing of the ITS region of several fungi (Takamatsu et al., 1998; Hirata and Takamatsu, 1996; Saenz and Taylor, 1999). ITS sequences from many of these organisms are available in GenBank. A further advantage of working with the ITS region is that several hundred copies of this region exist per individual cell, making it easier to amplify the region with PCR from small amounts of material (such as spores) than when using non-repeated regions of the genome (Lee and Taylor, 1990).

One of the methods for identifying unknown eukaryotic organisms is to perform PCR using non-specific primers which amplify part of the 18S gene or ITS sequence of nuclear rDNA genes followed by cloning and sequencing the PCR-

product. Cultivation-independent genetic detection of fungal populations directly in DNA extracted from complex environmental samples could reduce the time and cost for monitoring and analysis (Schwarzenbach et al., 2007). Sundelin et al. (2009) developed a culture-independent PCR based protocol for the early detection of several fungal pathogens from a wide range of crops. The authors used shot-gun PCR and cloning procedure which successfully detected the presence of possible plant pathogens in a range of diseased plant material *viz.* blackberry, cherry, daisy and birch and plant parts: fruits and leaves.

Yuan et al. (2010) also conducted a culture-independent approach to analyze the endophytic fungal community. One of the major problems working with plant associated fungi is the presence of plant DNA when DNA is extracted directly from the plant parts. In many cases plant DNA might be amplified to high levels and the causal pathogen might be over looked if only present in low amounts. In this study, the co-amplification of plant DNA with the use of eukaryotic ITS1 and ITS4 primers designed by White et al. (1990) was non-problematic as the PCR-products were present in different clones. Similar observation was reported by Sundelin et al. (2009).

The PCR products of the present study were cloned in pGEM T-easy vector and transformed. The number of transformed clones was chosen with care. In this study, nineteen clones were chosen to find possible causal agents of the observed symptoms. The DNA was isolated by 'boiling lysis' method and PCR of the insert was done with each chosen clone. Differentiation of the different inserts in clones was done by performing restriction digestion with the amplicons and observing their band patterns on agarose gel. The distinct clones were selected for sequencing. Using this approach, several fungal strains were identified that were associated with the diseased leaves. The results of both morphological and molecular tests were analysed together for appropriate identification of the pathogens. Several strains of *C. gloeosporioides* and *Exobasidium* sp. were found to be present in the infected leaves of tea.

Other fungi such as *Fusarium solani*, *Sporobolomyces* sp. and *Neofusicoccum parvum* were also observed.

C. gloeosporioides is a well known pathogen with a wide host range in different vegetables and horticultural crops that help it to survive throughout the year (Jeffries et al., 1990; Denoyes and Bandry, 1995; Manandhar et al., 1995; Zulfiqar et al., 1996; Pandey et al., 1997; Gaikwad et al., 2005). In the area of the present study, climatic conditions were optimally favourable for *C. gloeosporioides* to infect host plant as rainfall and humidity were very high. Green (1998) while studying on the distribution and severity of foliar diseases of yam observed that anthracnose disease is severe under conditions of high rainfall and relative humidity. So it is of outmost importance to know the relationship of the fungal pathogen *C. gloeosporioides* with tea varieties and control strategies of the pathogen as well as the disease. *C. gloeosporioides* was isolated from infected tea leaves from the field and after verification of Koch's postulations, the fungus was identified by its ribosomal RNA gene sequence.

The degree of susceptibility or resistance of a particular variety to a pathogenic fungus is determined through its differential pathogenicity to different varieties. Similarly pathogenicity of different fungi to a particular plant variety give us information about different infecting ability of different pathogen. Pathogenicity of the isolated fungus *C. gloeosporioides* was tested following detached leaf inoculation technique. Dickens and Cook (1989) also used this method to detect resistance and susceptibility of *Camellia* plants against *Glomerella cingulata.* Microscopic study of the fungus revealed that the length of each single-celled conidium ranged between 13-16 µm while the breadth ranged between and 4-6 µm. The hyphae were septate, with the diameter ranging between 3 - 5 µm. Similar observation regarding conidial size and shape has been reported by several authors (Kuo, 1999 and Kumar et al., 2002). While studying on *C. gloeosporioides* causing yam anthracnose, Abang et al. (2002) reported that the conidia were hyaline with rounded apices and measured 15-18 µm long and 4-6 µm wide.

A successful disease manifestation involves compatible host pathogen interaction under suitable environmental conditions. The compatibility is decided by multiple factors contributed by both host and pathogen. A unique serological similarity between pathogen and compatible host involving one or more antigenic determinants has been noted by many authors. In plants, the susceptibility towards a pathogen seems to increase with an increase in similarity between the antigens. These antigens cross react with each other in experimental antigen-antisera reactions. The present study was undertaken to determine the presence and the level of cross reactive antigens (CRA) between 8 different tea varieties included in this study and the pathogen, *C. gloeosporioides* by ELISA.

Enzyme linked immunosorbent assay has become increasingly popular since its discovery and introduction as a diagnostic tool in a practical form. Several formats have been developed, each with the ultimate goal of detection of antibody-antigen reaction at the lowest possible concentrations. It has been clearly pointed out by several authors (Chakraborty and Saha, 1994; Kratka et al., 2002; Dasgupta et al., 2005) on the basis of their findings that indirect ELISA can serve as a useful technique to detect cross-reactive antigens, which determine the susceptibility or resistance of a host in a host parasite combination. Therefore, in order to screen the different varieties for susceptibility towards *C. gloeosporioides*, it was considered worthwhile to study cross-reactive antigens between *C. gloeosporioides* and tea varieties by using indirect ELISA format, since this technique is one of the most sensitive serological techniques to detect and quantify low concentration of antigen.

Eibel et al. (2005) developed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) by raising polyclonal antibodies against *Ustilago nuda* and barley plant. Dasgupta et al. (2005) performed ELISA between tea varieties and *Curvularia eragrostidis*, which revealed the presence of a certain minimum level of antigens for compatible host-pathogen interaction. Several workers have used ELISA for early detection of pathogens (Chakraborty et al., 1995; Ghosh and Purkayastha, 2003).

In the present work, investigations were done to determine the susceptibility and resistance of tea varieties towards *C. gloeosporioides* by testing the presence of common antigens between host and pathogen. Antigenic cross-reactivity was detected by indirect ELISA between *C. gloeosporioides* and susceptible tea varieties. The higher ELISA values indicated a similarity between antigens in compatible interactions, while low ELISA values indicated the antigenic difference. The presence of CRA was obvious in antigen preparations having a protein concentration of 20µg/ml and with an antiserum dilution of 1:100. These results are in agreement with those Dasgupta et al. (2005) and Saha et al. (2010). Dasgupta et al. (2005) also observed that, indirect ELISA readily detects CRA in semi-purified preparation of antigen at concentration of 20µgml^{-1} with antiserum dilution of 1: 125.

El-Komy et al. (2010) detected sensitivity of the antiserum of *Phytophthora infestan* antigen. In addition, Alba and DeVay (1985) observed that it was possible to detect CRA in antigen concentrations lower than 50µg/ml and antiserum dilution to 1:250. Chakraborty and Sharma (2007) reported the presence of CRA with the purified antigen preparation at 50µgml^{-1} from the different tea varieties and antiserum concentration at 40µgml^{-1} raised against basidiospores of *Exobasidium vexans*. Here, ELISA values showed that a low level of common antigens was always present between all combinations irrespective of the degree of susceptibility of a variety to the pathogen. They suggested that the presence of common antigens at a certain minimum levels was essential. Similar observation was recorded by Dasgupta et al. (2005) in screening of commercially cultivated varieties of tea for their susceptibility to *C. eragrostidis* and also by Saha et al. (2010) in determination of the level of CRA which was correlated to the pathogenicity of *C. gloeosporioides* in different eggplant varieties. Purkayastha and Banerjee (1990) also reported similar results in a study on cloxacillin-induced resistance of soyabean against anthracnose. Several other authors (DeVay and Adler, 1976; Chakraborty et al., 1995) demonstrated the involvement of CRA in various host–parasite combinations.

Comparative analysis between results of pathogenicity test and that of indirect ELISA suggests that susceptibility rated by observing visual appearance of symptoms correlated well with the level of CRA between host and pathogen as recorded by ELISA. The most susceptible varieties (TV-18 and TV-30), which produced maximum mean disease index value when inoculated with *C. gloeosporioides* and also showed maximum CRA levels during ELISA when cross-reacted with *C. gloeosporioides* antiserum. In the same way, the most resistant variety (TV-25) produced minimum mean disease index value as well as minimum level of CRA. Our results obviously support the fact that serological resemblance involving one or more antigenic determinants occurs among compatible host and pathogen. The more the antigenic similarity between host and pathogen, the more is the possibility of the pathogen to successfully infect the host and cause disease. There is some possibility that the absence of specific components in tea leaves that are antigenically cross-reactive to *C. gloeosporioides* renders the plant resistant. On the other hand, a resistance strain incapable of infecting tea plant lack certain surface components that cross-react antigenically with host. There is a strong possibility that these are directly involved in the initial recognition between host and pathogen and form a correct molecular interfacial structure that allows for specific adhesion of the invading microbe as discussed by Dazzo and Hubbell (1975).

Recognition of the host by the pathogen is the earliest step in any direct plant-microbe interaction. Biochemical approaches have identified chemical elicitors of host defense responses as well as their similar receptors from the host. These elicitors may be pathogen derived or they may be chemical elicitors which induce systemic resistance in plants, thus reducing their susceptibility (Tyler, 2002). Chakraborty and Purkayastha (1987) noticed an antigenic difference in the susceptible soybean cultivar (cv. soymax) after chemical induction of resistance and concomitant increase in phytoalexin (glyceollin) level was also evident which can confer resistance to *M. phaseolina*. Purkayastha and Banerjee (1990) have seen that the antibiotic, cloxacillin, induced resistance in soyabean plants against

anthracnose caused by *Colletotrichum dematium* var. truncata. The leaves of soyabean showed alterations in the antigenic pattern when compared with untreated leaves. A specific host antigen present in the untreated leaves but not present in treated leaves suggest a special role of this antigen in disease reaction.

A further probable functional role of CRA may be in the infection process where it may act indirectly as immunosuppressor by interfering with innate host defense machinery. Some plant genes have been identified (Vogel et al., 2004) which when mutated lead to resistance towards specific pathogens only and are thought to be responsible for pathogen growth and development in the host. Resistance in this mutant may be due either to the loss of a susceptibility factor or to the activation of a novel form of defense as the mutants did not require signaling through either the salicylic acid or jasmonic acid/ ethylene defense pathways (Vogel et al., 2004).

It is very clear that, the ELISA can be considered as an important tool to detect CRA, to determine their properties and to investigate their possible role in host-parasite interactions. The failure to detect CRA between compatible host parasite interactions by conventional serological techniques (Carrol et al., 1972) has been recognized to a number of factors such as the method of extraction of antigen, culture of micro- organisms, age of plant tissue and low titer of antigens (DeVay and Adler, 1976).

The present study demonstrated that the indirect ELISA method is a sensitive technique and reliable in detecting CRA between susceptible *Camellia sinensis* varieties and *C. gloeosporioides*. It may be possible to detect and quantify the presence of the pathogen in infected tissues even before the appearance of symptoms. ELISA technique therefore may be utilized for the quick and accurate determination of the commercially cultivated tea varieties for susceptibility towards the fungal pathogen. Therefore, the selection of resistant tea varieties for growing in farmers fields and cloned cuttings of resistant varieties as a best management strategy for long-term disease control would be easier.

3.1. Literature review

Plants naturally synthesize diverse group of secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which have great importance to human and plants. It has been estimated that 70-80% of total world population largely depends on traditional herbal medicine to meet their primary health care need (Hamayun et al., 2006). Plants have been proved as useful source of several antifungal molecules that are harmless and caring to the environment. The use of botanicals for control of foliar diseases have gained importance due to the recent global awareness on negative effect of chemical fungicides, such as development of resistance, associated resurgence in fungi, accumulation of fungicide residues in food chain, environmental pollution, health risks and high costs (Seth et al., 1998; Van den Bosch and Gilligan, 2008). There are certain advantages in the deployment of botanical pesticides. These are biodegradable, safe to non-target organisms, renewable and suit to sustainability of local ecology and environment. This has awakened new interest in natural products as a source for novel industrial plant protection strategies.

At the beginning of present study, a literature survey on the useful antifungal phytochemicals was done following which fourteen plants were selected for experimental works. A detail of the traditional uses of all these plants is summarized in table 3.1. The plants showing promising activity against the major fungal pathogens of tea were reviewed in detail for the range of their activity and the nature of bioactive components present. This includes *Clausena excavata*, *Ocimum sanctum*, *Piper betle*, *Polyalthia longifolia* and *Xanthium strumarium.* The purpose of this review is to give an overview of the natural constituents that have been isolated from these plants and the range of their biological properties with special emphasis on antifungal compounds.

Table: 3.1. Selected plants and their traditional use as folk medicine

Name of plants	Local name	Traditional Application and Biological Activity
Bidens pilosa Linn.	Spanish needle	Leaf extract for treatment of cough, laryngitis, headache, conjunctivitis, rheumatism, infection, digestive and stomach disorder including peptic ulcer. Flower is used in the treatment of diarrhoea, dysentery and stomach upset due to food poisoning.
Clausena excavata Burm. f.	Agnijal	Decoction of leaves is taken for hypotension, fever, malaria, muscular pain and indigestion.
Datura stramonium Linn.	Datura	Smoke of leaves is used for asthma, causes sleepiness. Roots are good for tooth-ache.
Datura innoxia Mill.	Safed dhatura, Indian-apple	Leaves are used as repellent and vermicide. Seed are grind and cooked in mustered oil to cure scabies. Also used in asthma, wound, malaria and Leishmaniasis.
Emblica offcinalis L.	Amla	Fruits used to promote longevity, enhance digestion, strengthen the heart, purify the blood benefit the eyes, stimulate hair growth and enhance intellect. Used against constipation, fever and cough.
Eucalyptus globulus	Eucalyptus	The essential oil of leaf is powerful antiseptic, used against coughs and colds, sore throats, and other infections. Used as mouthwash toothpaste. Leaf extracts used in an anti-bacterial, antioxidant and anti-inflammation deodorant.
Lantana camara L.	Raimuniya	Used in bronchitis, stomach problems, rheumatism and to clean teeth.
Leonurus sibiricus Linn.	Guma & Raktadron	Anti-inflammatory, anti-diarrhoea. Leaf extract used in hemorrhage, weakness.
Measa indica	Kramighana phal	Leaves used as an agent for clearing the throat/vocal cord for producing a melodious sound.
Ocimum sanctum	Tulsi	Leaf juice with honey is given for 3-7 days for cough and cold.1:1 ratio of Tulsi leaf and Neem leaf paste is very effective for diabetes.
Polyalthia longifolia Var. *pendula*	Ashok tree	Powder of stem bark mixed with curd and sugar and mixture is given orally thrice a day to cure diarrhoea. Stem bark is dried, powdered and given orally in the treatment of gout.
Piper betle Linn.	Paan	Leaf paste with *Accia catchu* bark paste massaged on the skin of children in maggots, Herbal dye, antipyretic, antioxidant, anticancer antiulcer, anti-inflammatory, pain reliever and immunomodulating.
Syzygium cumini	Jamun	Hypoglycaemic, diuretics, analgesic, anti-inflammatory, antiplaque, antimicrobial, antidiarrhoel, antioxidant and gastro-protective.
Xanthium strumarium L.	Chotagokhru	Dry fruits of *X. Strumarium* kept on dried stem of *Calotropis procera* are burnt and the smoke is inhaled.

3.1.1. Overview on *Clausena excavata*

3.1.1.1. Occurrence

Clausena excavata Burm. f. is a wild shrub, belonging to the Rutaceae family (Burkill, 1935). The plant with objectionable smell exists as a bush growing wild and is found throughout the area stretching from the Himalayas and China to Peninsular Malaysia (Manosroi et al., 2005). *Clausena* is a genus of about fourteen species of evergreen trees, occurring mostly in India and tropical Asia (Shier, 1983). The plant is easy to grow, free of pests and diseases as well as capable of withstanding heavy pruning (Swarbrick, 1997) and is cultivated in India, South China and Southeast Asia (Blasco, 1983).

3.1.1.2. Traditional uses

Clausena excavata is traditionally useful as folk medicine in the treatment of abdominal pain, snakebite and as a detoxification agent (Ridley, 1922; Wu and Furukawa, 1982). The decoction of the roots is drunk for bowel-complaints, chiefly colic (Ali et al., 2000). The flowers and leaves may be boiled and the decoction taken for colic. A decoction of leaves is also given after child birth (Grieve and Scora, 1980). *C. excavata* leaves are also used to cure cold and malaria (Wiart et al., 2004). The powdered rootstock of this plant is also reported to be used in the treatment of decayed teeth whereas its stem is given in colic with or without diarrhoea (Yoshida, 1996).

3.1.1.3. Biological activity

Bioactivity of different plant parts of *C. excavata* have been studied by several authors (Table 3.2). Guntupalli et al. (2012) found that methanol extract from leaves possessed antioxidant activities. Manosroi et al. (2003) studied the folklore extract of *C. excavata* used traditionally in Thailand along with aqueous and acetone extracts and found immunomodulating activity of the wood extract on mouse immune system which explained the traditional use of this plant in Thailand. Sakong et al. (2011) reported anti-tussive, anti-hyperglycemia and anti-rhinitis activity in 70% ethanol extracts from the leaves of this plant.

Table: 3.2. Crude phytoextracts of *Clausena excavata* and their biological activities

Phytoextracts	Plant Part	Biological activity	Reference
Methanol	Leaf extract	Antioxidant	Guntupalli et al., 2012
Acetone	Root	Cytotoxocity	Sharif et al., 2011
Aqueous extract	Wood	*In vivo* immunomodulating	Manosroi et al., 2005
Aqueous, acetone & Thai folklore extracts	Wood	*In vitro* immunomodulatory	Manosroi et al., 2003
Hot aqueous & acetone extracts	Wood	Immunomodulatory	Manosroi et al., 2004
70% ethanol	Leaf	Anti-tussive, anti hyperglycemia & anti-rhinitis	Sakong et al., 2011
Methanol	Stem	Oral Toxicity in rat	Puongtip et al., 2011

3.1.1.4. Phytochemical components

A large number of bioactive secondary metabolites, such as coumarins, alkaloids, limonoids and some essential oils have been isolated from different plant parts of *C. excvata* (Table 3.3). These metabolites were extracted and purified by different techniques and phytochemical structures of these compounds have also been elucidated by means of different spectroscopic methods. A list of the antifungal compounds is given in Fig. 3.1. The literature reports of the bioactive components of this plant are summarized below.

Table: 3.3. A list of phytochemicals of *Cluasena excavata* and their biological activity

Phytochemicals	Chemical class	Plant Parts	Biological activity	References
Nordentatin, Dentatin, Clausenidin & Clauszoline-J	Pyrano-coumarins	Root bark	Antibacterial, antimycobacterial, cytotoxicity, antiproliferation & programmed cell death.	Wu and Furukawa, 1982; Sunthitikawinsakul et al., 2003; Sharif et al., 2011; Arbab et al., 2013
clausenidin, *O*-methylmuconal, 3-formyl-2,7-dimethoxycarbazole & Clauszoline-*J*	Pyrano-coumarins & Carbazole alkaloid	Rhizome & root	Anti-HIV	Kongkathip et al., 2010
3-methoxycarbonylcarbazole, 3-formylcarbazole, Mukonal & 2-hydroxy-3-formyl-7-methoxycarbazole	Carbazole derivative	Rhizome	Antifungal	Sunthitikawinsakul et al., 2003
Xanthyletin & Clausenarin	Coumarins	Root Bark	Cytotoxicity	Sharif et al., 2011
Clausenolide-1-methyl ether	Limonoids	Stem	Cytotoxicity	Sharif et al., 2011
Clausine-TY, Clausine-H & Clausine-B	Carbazole alkaloid	Stem bark	Cytotoxicity	Yap et al., 2007
Clausine-B	Carbazole alkaloid	Stem bark	Antiproliferative	Zain et al., 2009
Clausine-E & 2,7-dihydroxy-3 formyl-1-(3'-methyl-2'-butenyl)carbazole	carbazole alkaloids	Leaves & stem	Anti-topoisomerase II	Xin et al., 2008
Essential oil	Essential oils	Twig	Insecticidal	Cheng et al., 2008
Clausenolide-1-ethyl ether	Limonoid	Rhizome & root	HIV-1 inhibitory activity	Sunthitikawinsakul et al., 2003
Clausines B, E, H, I & K	Carbazole	Stem	Inhibition of rabbit platelet aggregation	Wu et al., 1996
Clausine-D & F	4-prenylcarbazole alkaloids	Stem bark	Antiplatelet aggregation	Wu and Huang, 1992
Sansoakamine	Carbazole alkaloid	Stems	Anti-malarial	Lastra et al., 2005
Clausenamine-A	Biscarbazoles	Stem & root bark	Cytotoxic Activities	Zhang and Lin, 2000

3.1.1.4.1. Coumarins

Coumarins are a major group of components that occur in *C. excavata*. In a study on the chemical components of leaves led to the isolation and identification of 10 new furanone-coumarins named clauslactones A, B, C, D, E, F, G, H, I and J together with the carbazole, clauszoline M and a coumarin, umbelliferone. The new coumarins were found to contain a C_{10} terpenoid side chain with a furanone ϒ-lactone moiety (Ito et al., 2000). Two new O-terpenoidal coumarins named excavacoumarin A and B along with a known coumarin were isolated from the leaves of *C. excavata* collected in Xishuangbanna, Yunnan, China by He et al. (2000). In addition, the authors also isolated six new *O*-terpenoidal coumarins named excavacoumarins B, C, D, E, F and G from the aerial part of the plant.

Xin et al. (2008) reported three new coumarins containing a C_{10} terpenoid side chain, clauslactones R, S and T, together with 14 known coumarins that were isolated from the leaves and stems of *C. excavata*. Takemura et al. (2000) reported four new furanone-coumarins, clauslactones-N, O, P and Q from the leaves and twigs of the plant that was collected in Indonesia. Two new pyrano-coumarins, claucavatin-A and B were isolated and identified from the acetone extract of the root bark of *C. excavata* (Huang et al., 1997). Moreover, two novels type of naturally occurring bicoumarins, named cladimarins A and B were isolated from the branch of *C. excavata* collected in Indonesia (Takemura et al., 2004). In another study, new coumarins namely excavatins A-M were isolated from leaves of the plant that were collected from the National Park Cuc Phuong, Ninh Binh, Vietnam (Thuy et al., 1999). Sunthitikawinsakul et al. (2003) isolated several compounds including four known coumarins from *C. excavata* that were found to possess antimycobacterial activity and demonstrated no cytotoxicity against KB and BC-1 cell lines.

3.1.1.4.2. Alkaloids

Carbazole alkaloids are a major component of *C. excavata*, which have been extracted from its different parts. Wu et al. (1999) isolated and identified ten new carbazole alkaloids, clausine M, N, O, P, Q, R, S, U, V and clausenatine-A, together with 39 known compounds from the acetone extract of the root bark of *Clausena excavata*. Yap et al. (2007) found a new carbazole alkaloid, 3-carbomethoxy-2-hydroxy-7-methoxycarbazole, Clausine-TY, along with two known carbazole alkaloid, Clausine-H and Clausine-B from the ethyl acetate extract of the stem bark from Malaysia. Kongkathip et al. (2010) isolated three carbazole compounds, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, and one coumarin Clausenidin from the rhizomes and roots. A new carbazole alkaloid, sansoakamine, together with 11 known compounds was isolated from the stems of *C. excavata* (Sripisut and Laphookhieo, 2010). Zhang and Lin (2000) reported biscarbazoles, clausemine-A from the stem and root bark extracts. Clausemine-A and other two structurally related biscarbazoles showed potent cytotoxic activities against a variety of human cancer cell lines *in vitro*.

3.1.1.4.3. Other constituents

Sunthitikawinsakul et al. (2003) reported the isolation of Limonoid 1, named as clausenolide-1-ethylether from the crude ethanol extract of the rhizomes and the roots *C. excavata.* Cheng et al. (2008) demonstrated that essential oils present in the leaf and twig of *C. excavata* possess mosquito larvicidal activity. The individual components were able to inhibit the growth of *Aedes aegypti* and *A. albopictus* larvae at a low concentration. The essential oils were obtained from hydrodistillation and their constituents were determined by GC-MS analyses. The results of larvicidal assays showed that, the effective constituents in leaf and twig essential oils were limonene, *γ* -terpinene, terpinolene, *β*-myrcene, 3-carene and *p*-cymene. He et al. (2002) reported the isolation of five new tetranor triterpenoids from the aerial part of *C. excavata*.

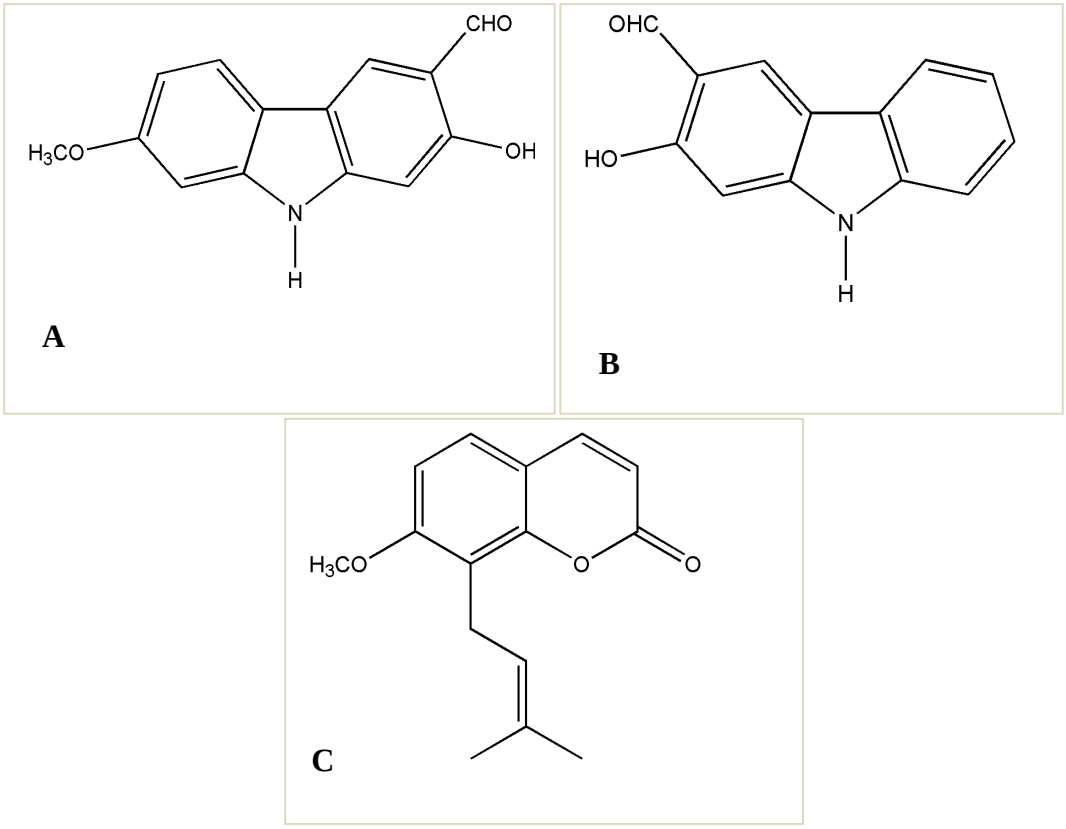

Fig.3.1. Structure of antifungal phytochemical compounds from *Clausena excavata.*

A: 2-hydroxy-3-formyl-7-methoxycarbazole (Sunthitikawinsakul et al., 2003).

B: Mukonal (Sunthitikawinsakul et al., 2003).

C: Osthol (Thongthoom et al., 2010).

3.1.2. Overview on *Xanthium strumarium*

3.1.2.1. Occurrence

Xanthium strumarium L. belonging to Asteraceae family is an annual herb with a short, stout, hairy stem. The plant commonly found as a weed is widely distributed in North America, Brazil, China, Malaysia and hotter parts of India. The whole parts of this plant have been used as traditional medicine. The genus *Xanthium* includes 25 species; two species of *Xanthium, X. indicum* and *X. strumarium* have been reported in India (Gupta and Moupachi, 2013). All 25 species found in American origin of which *Xanthium spinosum* and *X. strumarium* were used medicinally in Europe, North America and *X. strumarium* in China and Malasiya (Kamboj and Saluja, 2010).

3.1.2.2. Medicinal use

The whole plant, specially root and fruit is used as medicine. According to Ayurveda, *X. strumarium* is cooling, laxative, fattening, tonic, digestive, antipyretic and improves appetite, voice, complexion and memory. It cures leucoderma, biliousness, poisonous bites of insects, epilepsy, salivation and fever. The plant of *Xanthium* yields xanthinin which acts as a plant growth regulator. Antibacterial activity of xanthinin has also been reported. Seed yields semi-drying edible oil (30-35%) which resembles sunflower oil and used in bladder infection, herpes and erysipelas (Srinivas et al., 2011). *X. strumarium* is also reported to be anti-ulcerogenic, antitrypanosomal, anti-helmintic, anti-inflammatory and possess antimicrobial activities. Besides, it shows a diuretic action and a hypoglycaemic action and also possess anti-leishmanial and antifungal activities (Kamboj and Saluja, 2010).

3.1.2.3. Phytochemical constituents and biological activities of *Xanthium strumarium*

Several constituents exhibiting a wide range of bioactive properties have been reported to be isolated from the extracts of *X. strumarium*. A list of the different

phytochemicals isolated from this plant and their biological activities are listed in table 3.4. Additionally, the reported chemical structure of antifungal compounds (Fig. 3.2) reported from this plant. Sesquiterpenoids are the largest group of natural terpenoids and contains several compounds. Sesquiterpenoid lactones are particularly characteristic of the Asteraceae family, although occurs in other plant families. The phytochemical taxonomic studies showed that, this plant is a rich source of sesquiterpens. Ramirez-Erosa et al. (2007) isolated two sesquiterpene, xanthatin and xanthinosin from the burs of *X. strumarium* and evaluated *in vitro* cytotoxicity on the human cancer cell lines which revealed moderate to high *in vitro* cytotoxic activity in the human cancer cell lines WiDr ATCC (colon), MDA-MB-231 ATCC (breast) and NCI-417 (lung). Moreover, Peris et al. (2008) investigated bactericidal and fungicidal activity of Xanthatin against *Colletotrichum gloeosporioides, Trichothecium roseum, Bacillus cereus* and *Staphylococcus aureus*. Mahmoud (1998) reported three new xanthanolide and xanthane type sesquiterpenoids, 11α, 13-dihydroxanthatin, 4ß,5ß-epoxyxanthatin-1α,4α-endoperoxide and 1ß,4ß,4α,5α-diepoxyxanth-11(13)-en-12-oic acid, together with seven known compounds from the aerial part of *X. strumarium*. Two xanthanolide sesquiterpene lactones, 8-*epi*-xanthatin and 8-*epi*-xanthatin epoxide were reported to be isolated from the leaves *of the plant by* Kim et al. (2003).

A new xanthanolide diol derivative, 11α, 13-dihydroxyxanthatin and a new dimeric xanthanolide sesquiterpene lactone, pungiolide-C, in addition to some known compounds were isolated from aerial parts of *Xanthium strumarium* and fruit of X. *pungens* by Ahmed et al. (1999).

Apart from sesquiterpenes, other components were also found in this plant which was reported as bioactive. Ma et al. (1998) isolated a new thiazinedione and four known phenolic compounds from the aqueous acetone extract of the fruit. The phenolic compounds were identified as caffeic acid, potassium 3-O-caffeoyl quinate, 1, 5-di-O-caffeoylquinic acid and 1,3,5-tri-O-caffeoylquinic

acid. Han et al. (2006) reported the isolation of two new thiazinediones along with five known compounds from the fruits of *X. strumarium*. The structures of new compounds were determined to be 7-hydroxymethyl-8,8-dimethyl-4,8-dihydrobenzol[1,4] thiazine-3,5-dione-11-O-ß-D-glucopyranoside and 2-hydroxy-7-hydroxymethyl-8,8-dimethyl-4,8-dihydrobenzol[1,4]thiazine-3,5-dione-11-O-ß-D-glucopyranoside. The other five known compounds were identified as xanthiazone, chlorogenic acid, ferulic acid, formononetin and ononin. In another study, Han et al. (2007) isolated and identified ten caffeoylquinic acids and three heterocyclics from the active polar n-butanol fraction of *X. strumarium*. The isolated caffeoylquinic acids could partially explain the antinociceptive effect of *X. strumarium* polar extract.

Islam et al. (2009) purified and characterized a total of four compounds as stigmasterol, 11-hydroxy-11-carboxy-4-oxo-1(5), 2(Z)-xanthadien-12, 8-olide, daucosterol and lasidiol-10-anisate. They also evaluated their toxicity on rats; the methanol extract of seedlings showed mortality, while both seedling and mature plant extracts raised the serum alanine transaminase and aspartate transaminase values and produced significant abnormalities in the histopathology of liver and kidney of rats. On the other hand, the aqueous soluble fraction of methanol extract of mature plant and methanol crude extract of seedlings demonstrated a significant toxicity in the brine shrim lethality bioassay.

Scherer et al. (2009) evaluated the antimicrobial activity of *X. strumarium* leaf extracts against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa* and *Clostridium perfringens*. They also investigated the presence of the toxic compound carboxyatractyloside in different plant parts. The carboxyatractyloside was found in cotyledons and seeds, but not in adult leaves and burrs. The authors suggested that *Xanthium strumarium* leaves only in adult stage can be used for medicinal purposes.

Pandey and Rather (2012) isolated and identified caffeic acid, xanthiazone and xanthiazone-(2-O-caffeoyl)-ß-D-glucopyranoside from ethyl acetate fraction

and methanol extract of *X. strumarium* by repeated column chromatography over silica gel and sephadex LH20. Wahab et al. (2012) observed that the ethyl acetate extract of the aerial parts of *X. strumarium* have a pentacyclic triterpene, 3ß-acetoxy lup-20(29)-ene, two sterols, stigmasterol and ß-sitosterol and a fatty acid, palmitic acid.

Table: 3.4. List of phytochemicals and their biological activities from *Xanthium strumarium*

Phytochemicals /Phytoextracts	**Plant parts**	**Biological activities**	**References**
8-*epi*-tomentosin	Different plant parts	Cytotoxicity	Ahn et al., 1995
Tomentosin, Xanthumin & 8-*epi*-xanthatin-1ß,5ß-epioxide	Aerial parts	Antimalarial	Joshi et al., 1997
8-*epi*-xanthatin	Aerial parts	Cytotoxicity	Ahn et al., 1995
Deacetyl xanthumin	Leaves	Antifungal	Kim et al., 2002
Xanthatin	Different plant parts	Antimicrobial, cytotoxicity & antiinflammatory	Ahn et al., 1995; Scherer et al., 2009
Cinnamic acid	Leaves	Antimicrobial	Scherer et al., 2009
Xanthinosin	Aerial	Cytotoxicity	Ramirez-Erosa et al., 2007
Essential oil	Leaves	Antimicrobial & antioxidant	Scherer et al., 2011
Alcoholic extract	Leaves	Antifungal & Antibactrial	Khuda et al., 2012
50% ethanolic extract	Leaves	Antitrypanosomal	Talakal et al., 1995

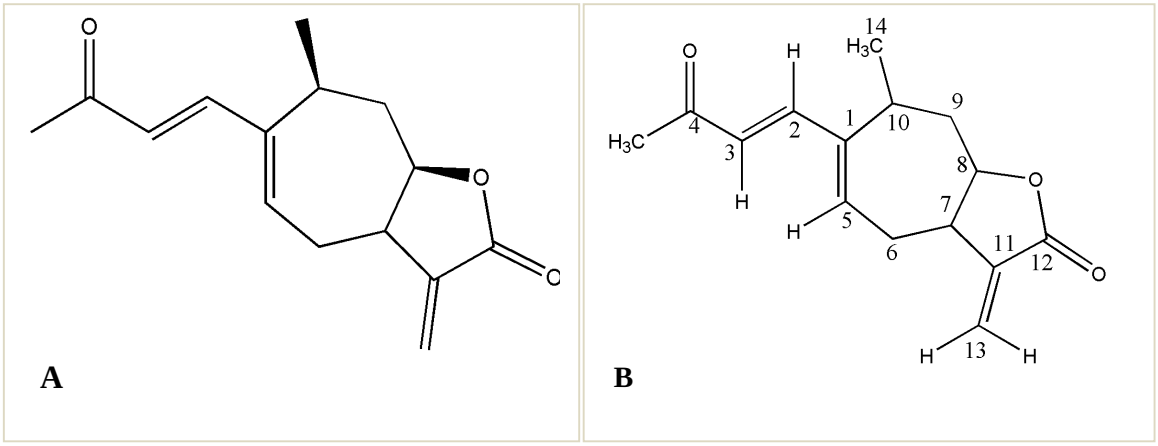

Fig. 3.2. Structure of antifungal phytochemical compounds from *Xanthium strumarium*

A: Deacetyl-xanthumin (Kim et al., 2002).

B: Xanthatine (Lavault et al., 2005; Saha et al., 2012a).

3.1.3. Overview on *Piper betle*

3.1.3.1. Occurrence

Piper betle Linn. is a tropical plant closely related to common pepper and belongs to the Piperaceae family. It is extensively grown in India, Srilanka, Malayasia, Thailand, Taiwan and other Southeast Asian countries (Bajpai et al., 2010). It is one of the important plants in the Asiatic region which ranks second to coffee and tea in terms of daily consumption. In recent years several reports have been published on the effects of the plant extract and chemical constituents on different biological activities *in vitro* and *in vivo*.

3.1.3.2. Medicinal use

P. betle has been extensively used in traditional herbal remedies in India, China, Taiwan, Thailand and many other countries. It is reported for various pharmacological activities such as antifungal, antimicrobial, antioxidant, antimutagenic, anticarcinogenic, antiinflamatory etc. (Ali et al., 2010). The leaf extract, fractions and purified compounds are found to play a role in oral hygiene, anti-diabetic, cardiovascular, anti-inflammatory, immunomodulatory, anti-ulcer, hepato-protective and anti-infective etc. (Kumar et al., 2010). The chemicals constituent of essential oil consists of mainly terpens and phenols. The characteristic flavour of betle is due to the betle phenols. The terpenoids include 1, 8-cineole, cadinene, camphene, caryophylline, limonene, pinene etc. Chavicol, allyl pyrocatechol, carvacrol, safrole, eugenol and chavibetol are the major phenol found in *Piper betle* (Bajpai et al., 2010). A number of biologically active compounds from *P. betle* have potential for use as medicines, neutraceuticals and industrial compounds. It is also used in drug delivery through buccal mucosa by passing the gastric route (Kumar et al., 2010).

3.1.3.3. Phytochemical investigation of *Piper betle* extracts

Piper betle has been found to possess several phytochemicals with a wide range of bioactive properties (Table 3.5). The plant is a rich source of less polar

phenolic compounds. The solvents used for extraction affected the concentration of total phenolic content in extracts. Ethyl acetate was found to be the best for the extraction of antioxidant compounds from *P. betle* leaves due to its nonpolar components (Maisuthisakul, 2012). Evans et al. (1984) isolated and identified five propenylphenols, chavicol, chavibetol, allylpyrocatechol, chavibetol acetate and allylpyrocatechol diacetate from chloroform extract of *Piper betle* (Piperaceae) leaves. The five propenylphenols showed significant fungicidal and nematocidal activity.

Amonkar et al. (1986) reported phenolic compounds eugenol and hydroxychavicol from leaf extracts. Ghosh and Bhattacharya (2005) characterized aristololactam A-II and a new phenyl propene as 4-allyl resorcinol from the alcoholic extract of *P. betle* roots, while the petroleum ether extract yielded a diketosteroid, stigmast-4-en-3, 6-dione. Bhattacharya et al. (2007) evaluated healing property of the *P. betle* phenol, allylpyrocatechol against the indomethacin-induced stomach ulceration in the rat model and found that it correlates with its antioxidative and mucin protective properties. Yin et al. (2009) isolated and identified nine compounds as 6ß-hydroxystigmast-4-en-3-one, ß-sitosterol, stigmasterol, oleanolic, 23-hydroxyursan-12-en-28-oic acid, ß-sitosterol-3-O-ß-D-glucoside-6'-O-palmitate, ß-daucosterol, (2S)-4'-hydroxy-2,3-dihydroflavonone-7-O-ß-D-glucoside and α-ethylglucoside from the petroleum ether and ethyl acetate soluble fractions of the 70% acetone extract of the stem.

Ali et al. (2010) isolated hydroxychavicol from leaf extract of *P. betle* and investigated it for antifungal activity. It exhibited inhibitory effect on fungal species of clinical significance, with the MICs ranging from 15.62 to 500 µg/ml for yeasts, 125 to 500µg/ml for *Aspergillus* species and 7.81 to 62.5µg/ml for dermatophytes.

Table: 3.5. A list of phytochemicals from *Piper betle* and their biological activities

Phytochemicals	**Plant part**	**Biological activities**	**References**
Chavicol, Chavibetol acetate & Allylpyrocatechol diacetate	Leaves	Antifungal & nematocidal	Evans et al., 1984; Paranjpe et al., 2013
Hydroxychavicol	Leaves	Xanthine oxidase inhibitor	Murata et al., 2009
Chavibetol & Allylpyrocatechol	Leaves	Antimicrobial	Ramji et al., 2002
		Photosensitization-mediated lipid peroxidation inhibition	Mula et al., 2008
		Antioxidant & radioprotection of pBR322 DNA.	Bhattacharya et al., 2007; Rathee et al., 2006
		Antibacterial, antioxidant, anti-inflammatory & anticancer	Sharma et al., 2009
		Antifungal & anticancer	Ali et al., 2010
Essential oil	Leaves	Antifungal & antibacterial	Garg and Jain, 1992
Fatty acid (Stearic & Palmitic) & Hydroxyl fatty acid esters (Stearic acid, Palmitic & Myristic)	Leaves	Antifungal & antimicrobial	Nalina and Rahim, 2007
Essential oil	Seed	Anti-leishmanial	Misra et al., 2009
		Antibacterial	Sharma et al., 2009
	Leaves	Antibacterial & antifungal	Sugumaran et al., 2011; Caburian and Osi, 2010
		Larvicidal	Wardhana et al., 2007
Anticoagulant compounds	Leaves	Anticoagulant	Jesonbabu et al., 2012

Bajpai et al. (2010) investigated phytochemicals in eight cultivars of *P. betle* leaves and detected the presence of chavicol, allylpyrocatechol, chavibetol, chavicol acetate, allylpyrocatechol acetate, chavibetol acetate and allylpyrocatechol diacetate.

Dwivedi and Mehta (2011) isolated and characterized four aliphatic compounds Pentadecyl 6-hydroxytridecanoate, Pentatriacontanol, Methyl hexacos-7-enoate and 6, 9-heptacosa diene in pure form from hexane fraction of *P. betle* leaf stalk. Lin et al. (2013) reported a new hydroxychavicol dimer, 2-(ϒ-hydroxychavicol)-hydroxychavicol from the roots along with five known compounds, hydroxychavicol, aristololactam A II, aristololactam B II, piperolactam A and cepharadione A.

3.1.3.4. Biological activity

A wide range of biological activity has been reported from *P. betle* crude and fractionated extracts (Table 3.6). Evans et al. (1984) reported five propenylphenols as chavicol, chavibetol, allylpyrocatechol, chavibetol acetate and allylpyrocatechol diacetate with significant fungicidal and nematocidal activity from the chloroform extract of leaves. The fungicidal activities of new compounds chavibetol acetate and allylpyrocatechol diacetate were found to be in the same range as the well-known natural fungicides eugenol and catechol. The common practice of chewing the leaves of *P. betle* may extract these allyl phenols and inhibit common opportunistic fungal pathogens such as *Candida albicans*, which can infect the oral mucosa, since, leaves of *P. betle* have been used as dressings for sores and wounds (Bangar et al., 1966). Nazmul et al. (2011) performed antifungal susceptibility test against five fungi and found that the best anti-fungal activity was evident against *Trichophyton rubrum*. A list of the antifungal components is shown in Fig. 3.3. Harsha et al. (2012) investigated the biomolecule protective, *in vitro* antioxidant and anti-anxiety properties of *P. betle* leaf extract. The hydro-alcohol extract was examined for radical scavenging ability against 2, 2-diphenyl-2-picryl hydrazyl (DPPH), 2, 2'-azino-bis

3- ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, metal chealating inhibition, DNA and protein damage protection assay, anti lipid peroxidation, reducing power and nitric oxide radical scavenging activities. The plant extract provided protection against DNA and protein damage and this was comparable to gallic acid, the standard.

Table:3.6. A list of phytocextracts from *Piper betle* and their biological activities

Phytoextracts	Plant parts	Biological activities	References
Ethanol & ethyl acetate	Leaf stalk	Antimicrobial	Shitut et al., 1999
		In vitro antioxidant	Shah et al., 2013
Ethanol extract	Leaves	Antioxidant	Rathee et al., 2006; Arambewela et al., 2006
		Antimicrobial	Reveny, 2011; Datta et al., 2011
Aqueous extract	Leaves	Antioxidative & antiplatelet	Lei et al., 2003
		Acetylcholinesterase inhibitor	Das and De, 2011
		Antifungal	Himratul-Aznita et al., 2011
		Antihaemolytic & antioxidative	Chakraborty and Shah, 2011
		Antibactrial	Subashkumar et al., 2013
Methanol extract	Leaves	Antifungal & antibactrial	Nazmul et al., 2011; Nair and Chanda, 2008
		Antihyperglysemic & antinociceptive	Shanthun-Al-Arefin et al., 2012
		Antimutagenic, antitumor & antioxidant	Paranjpe et al., 2013; Manigauha et al., 2009
Crude extract	Leaves	Cytotoxicity	Sundeep et al., 2011
Methanol, ethyl acetate & petroleum ether	Leaves	Antibacterial, antihaemolytic & antioxidant	Chakraborty and Shah, 2011
Ethanol, pet ether & chloroform	Leaves	Antibacterial	Ali et al., 2012
Hydroalcohol extract	Leaves	Biomolecules protective & antioxygenic	Harsha et al., 2012

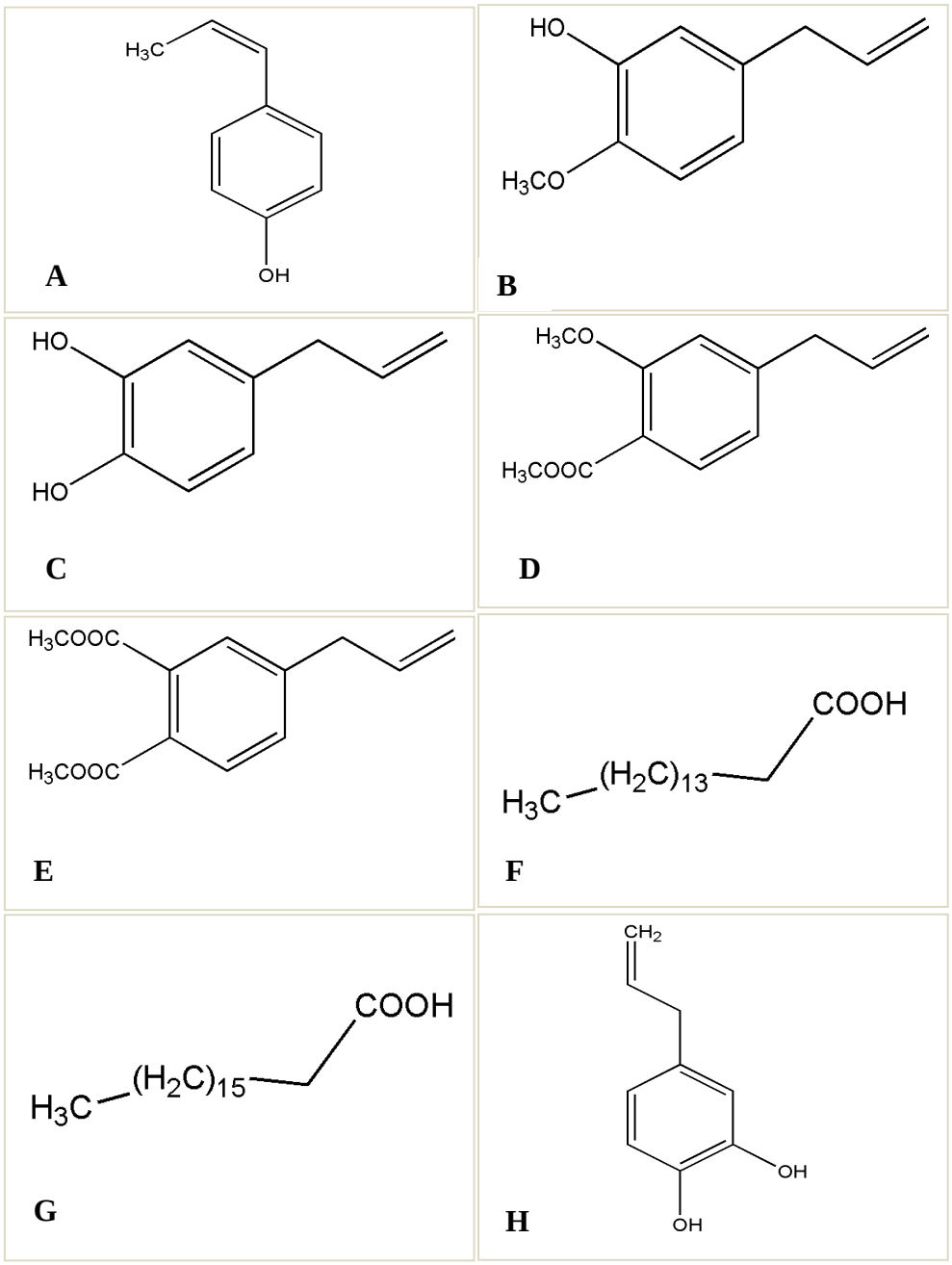

Fig.3.3. Structure of antifungal phytochemical compounds from *Piper betle*

A: Chavicol, **B:** Chavibetol, **C:** Allypyrochatechol, **D:** Chavibetol acetate & **E:** Allylpyrocatechol diacetate (Evans et al., 1984)

F: Palmitic acid and **G:** Stearic acid (Nalina and Rahim, 2007)

H: Hydroxychavicol (Ali et al., 2010).

3.1.4. Overview on *Ocimum sanctum*

3.1.4.1. Occurrence

Ocimum sanctum L. (Lamiaceae) is a shrub reaching a height of 0.5 to 1.5m. The plant is locally known as Tulsi and English name is Holy Basil (Rahman et al., 2009). In India Tulsi is taken as the most sacred plant. It is grown in tropical and sub-tropical regions including India. It is also abundantly found in Malaysia, Australia, West Africa and some of the Arab countries. *Ocimum sanctum* is the most prominent species of the genera. Morphologically it is an erect, much branched about 30-60 cm tall with hairy stems and simple opposite green leaves that are strongly scented. Leaf color ranges from light green to dark purple. The leaves of the plant are considered to be very holy and often form a consistent part of the Hindu spiritual rituals (Mondal et al., 2009; Bhuvaneswari and Jegatheesan, 2011).

3.1.4.2. Medicinal use

O. sanctum is known as “Queen of plants” or “The mother medicine of nature”. The plant is considered to possess enormous properties for curing and preventing diseases. Various parts of the plant are used for the treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite etc. Decoction of leaves is suggested for malaise and cough in colds. The plant has also been suggested to possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic and diaphoretic actions (Prakash and Gupta, 2005). *O. sanctum* is an important botanical supplement used in combination with other plants for the treatment of various stress-induced disorders in India and other Asian countries. Numerous studies have shown the immunomodulatory and antistress potential of *O. sanctum* leaf extracts (Mediratta et al., 2002).

3.1.4.3. Phytochemical constituents of *Ocimum sanctum*

Several bioactive molecules and nutrients have been found in *O. sanctum* (Table 3.7). The quantity of these constituents depends on the nature of soil, harvesting, processing and storage techniques. Zheljazkov et al. (2008) evaluated the biomass productivity, oil content and oil composition of sweet basil (*Ocimum basilicum* L.) and holy basil (*Ocimum sanctum* L.) at four locations in Mississippi. The main constituent of sweet basil cultivars was linalool with other constituents being camphor, α-humulene, eucalyptol, eugenol, bornyl acetate, methyl chavicol, trans-carvophyllene, α-trans-bergamotene, and cadinol. The main constituents of basil were methyl chavicol, eugenol and eucalyptol with other constituents being α-humulene, humulene-epoxide II, trans-caryophyllene, α-trans-bergamotene and Υ-cadinene.

Rahman et al. (2009) isolated ß-sitosterol-D-glycoside from the petroleum ether extract of the leaves of *O. sanctum* and its structure was elucidated by the help of UV, IR, ^{1}H-NMR and ^{13}C-NMR spectral data. GC-MS analysis of hydroalcoholic extract of *O. sanctum* leaves by Devendran and Balasubramanian (2011) lead to identification of 10 compounds that included eugenol (43.88%), caryophyllene (26.53%), cyclopentane, cyclopropylidene-(1.02%), cyclohexane, 1,2,4-triethenyl (15.31%), octadecane, 1,1-dimethoxy-(2.04%) and benzene methanamine, N,N,a,4-tetramethyl-(2.04%).

Table:3.7. A list of phytochemicals from *Ocimum sanctum* and their biological activities.

Phytochemical components	Plant part	Biological activity	References
Cirsilineol, isothymonin, rosmarinic acid & appreciable quantities of eugenol	Leaves & stem	Antioxidant & cyclooxygenase -1 inhibitory	Kelm et al., 2000
Isothymusin		Antioxidant	
Apigenin & cirsimaritin		Cyclooxygenase -1 inhibitory	
Eugenol	Leaves	Anthelmintic	Asha et al., 2001
Ocimumosides A & B, ocimarin, apigenin-7-O-D-glucuronic acid & 4-allyl-1-O-D-glucopyronosyl-2-hydroxybenzene	Leaves	Antistress	Gupta et al., 2007
6-allyl-3',8-dimethoxy-flavan-3,4'-diol, 6-allyl-3-(4-allyl-2-methoxyphenoxy)-3',8-dimethoxyflavan-4'-ol, 5-allyl-3-(4-allyl-2-methoxyphenoxymethyl)-2-(4-hy-droxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran, 1,2-bis(4-allyl-2-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane, 1-(4-hydroxy-3-methoxyphenyl)-1,2,3-tris(4-allyl-2-methoxyphenoxy)propane, 1-allyl-4-(5-allyl-2-hydroxy-3-methoxyphenoxy)-3-(4-allyl-2-methoxyphenoxy)-5-methoxybenzene & 3-(5-allyl-2-hydroxy-3-methoxyphenyl)-1-(4-hydroxy-3-methoxyphenoxy)-prop-1-ene	Leaves	Leishmanicidal	Suzuki et al., 2009
Flavanoids	Leaves	Antibacterial	Ali and Dixit, 2012;
Orientin & vicenin	Leaves	Antioxidant	Devi et al., 2000
Rosmarinic acid	Leaves & stem	Antioxidant	Hakkim et al., 2007
Essential oils	Leaves	Antimicrobial & antifungal	Dharmagadda et al., 2005; Khan et al., 2010

Cont..

(Contd...) A list of phytochemicals from *Ocimum sanctum* and their biological activities.

Phytochemical components	**Plant part**	**Biological activity**	**References**
Essential oils	Aerial part	Antioxidant	Trevisan et al., 2006; Bunrathep et al., 2007
Fixed oil & linolenic acid	Seed	Anti-inflammatory, antipyretic, hypotensive, anticoagulant & immunomodulatory	Singh and Majumdar, 1997; Singh et al., 2007
		Antibacterial	Singh et al., 2005
		Antidiabetic, antihypercholesterolaemic & antioxidant	Gupta et al., 2006
Essential oil & its major component, eugenol	Leaves	Antifungal & antiaflatoxigenic	Kumar et al., 2010
Polysaccharides		Antioxidant & radioprotective	Subramanian et al., 2005
Benzene & chloroform extracts		Antimicrobial	Baskaran, 2008
Methanol extract	whole plant	Antimicrobial	Bhatt et al., 2012
Aqueous, methanol & various solvent extracts	Leaves & root	Antibacterial	Goyal and Kaushik, 2011
		Antimicrobial & immunostimulant	Singh et al., 2013; Godhwani et al., 1988
		Antioxidant & antibacterial	Prasad et al., 2012

3.1.5. Overview on *Polyalthia longifolia*

3.1.5.1. Occurrence

Polyalthia longifolia belongs to the family Annonaceae. It occurs mainly in India, Africa, Asia, Australia and New Zealand. The plant is locally known as Ashoka **(**Danlami et al., 2011). It is a high evergreen tree, narrow branching, about 25-60 ft high with long green leaves (7-15 cm in length) and round or oval shaped fruits (Sharker and shahid, 2010).

3.1.5.2. Biological activity

Literature survey revealed that various parts of the plant possess different biological activities. A number of biologically active compounds have been isolated from this plant. The isolated compounds were studied for various biological activities like antibacterial against pathogenic bacteria viz. *Bacillus subtilis*, *Sarcina lutea*, *Xanthomonas compestris*, *Escherichia coli, Klebsiella pneumonea* and *Pseudomonas* sp. The MIC was found against the tested bacteria ranging from 21.00 to 44.20mm. The highest zone of inhibition produced by the hexane, methanol and chloroform extracts of *Polyalthia longiflia* at a concentration of 500µg/10µl against pathogenic bacteria i.e. *Sarcina lutea* were found 41.80mm, 44.20mm and 43.50mm respectively (Parvin et al., 2013). The antiulcer activity of ethanol extract against aspirin plus pylorous ligation induced gastric ulcer in rats. The pylorous ligation exhibited significant reduction in gastric volume, free acidity and ulcer index as compared to control. It also showed 89.71% ulcer inhibition in HCl-ethanol induced ulcer and 95.3% ulcer protection index in stress induced ulcer (Malairajan et al., 2008). Misra et al. (2010) were isolated a compound, 16α-Hydroxycleroda-3,13 (14)Z-dien-15,16-olide which showed as a non-cytotoxic, orally active antileishmanial activity and it also inhibited recombinant DNA topoisomerase-I which ultimately induced apoptosis.

3.1.5.3. Medicinal use

Almost all parts of the plant are used in the Indian traditional system of medicine for the treatment of various ailments in human beings. This plant is used as an antipyretic agent in indigenous systems of medicine. *P. longifolia* is used in the treatment of colitis, diarrhea, anorexia, skin diseases, sore throat, cough and colds (Sharker and shahid, 2010). Besides, it is also used for the treatment of fever, mouth ulcers, hypertension, helminthiasis, gonorrhea, uterine ailments, leucorrhoea and menorrhagia (Parvin et al., 2013).

3.1.5.4. Phytochemical constituents of *Polyalthia longifolia*

Phytochemical studies of *Polyalthia longifolia* leaves, bark, roots, root bark and seeds have revealed the occurrence of various types of diterpenoids, triterpenes, alkaloids and some essential oils with numerous biological activities (Table 3.8). A list of chemical constituents with antifungal properties is depicted in Fig. 3.4.

Hara et al. (1995) isolated nine new clerodane and *ent*-halimane diterpenes from hexane extract of the stem bark of *P. longifolia.* The structures of these compounds were identified as 16-hydroxycleroda-4(18),13-dien-16,15-olide, 16-oxocleroda-4(18),13*E*-dien-15-oic acid, cleroda-4(18),-13-dien-16,15-olide, 16-hydroxy-*ent*-halima-5(10),13-dien-16,15-olide, 16-oxo-*ent*-halima-5(10),13*E*-dien-15-oic acid, *ent*-halima-1(10),13*E*-dien-16,15-olide, 16-oxo-*ent*-halima-5(10),13*E*-dien-15-oic acid, *ent*-halima-5(10),13-dien-16,15-olide and *ent*-halima-5(10),13*E*-dien-16,15-olide. Other five known clerodane diterpenes were also isolated.

Chen et al. (2000) reported a new halimane diterpene, 3β,5β,16α-trihydroxyhalima-13(14)-en-15,16-olide and a new oxoprotoberberine alkaloid, (-)-8-oxopolyalthiaine, along with 20 known compounds that were isolated from a methanolic extract of the plant.

Islam et al. (2001) reported the isolation of two antimicrobial diterpenes; Kolavenic acid and 16-oxo-cleroda-3,13(14)*E*-dien-15-oic acid from the petroleum ether extract of the seed of *P. longifolia.* Chang et al. (2006) isolated and

characterized a new clerodane diterpenoid 16-hydroxycleroda-13-ene-15,16-olide-3-one from the bark along with 23 known compounds and phytosteroids.

Ogunbinu et al. (2007) studied the essential oils of the leaf and stem bark of *P. longifolia* by means of gas chromatography and gas chromatography/mass spectrometry. The leaf oil was found to be almost exclusively composed of sesquiterpene derivatives, being represented by allo-aromadendrene, caryophyllene oxide, ß-caryophyllene, ß-selinene, α-humulene and arcurcumene. However, α-copaene and α-muurolol, ß-selinene, viridiflorene, α-guaiene, allo-aromadendrene and δ-cadinene were the major constituents in the oil of the bark sample. All the other sesquiterpenoid compounds were observed in amount greater than 1%. α-Pinene and camphene, which are the 2 monoterpenoids present in the leaf oil, could not be detected from the bark essential oil.

Lee et al. (2009) reported the isolation of three new clerodane type diterpenes, 6α,16 dihydroxycleroda-3,13-dien-15-oic acid, 6α,16-dihydroxycleroda-4(18),13-dien-15-oic acid and 4α,18ß-epoxy-16-hydroxyclerod-13-en-15-oic acid, and four new protoberberine alkaloids together with 11 known substances from methanol extract of the stems of *P. longifolia* var. pendula. A new Υ-methoxybutenolide clerodane diterpene, 16α-methoxycleroda-3, 13Z-dien-16, 15-olide were isolated from the petroleum ether extract of the bark of the plant. Sashidhara et al. (2009) reported the isolation of diterpene (−)-3α,16α-dihydroxycleroda-4(18),13(14)*Z*-dien-15,16-olide which was a new compound. Another constituent, (−)-3ß,16α-dihydroxycleroda-4(18), 13(14)*Z*-dien-15,16-olide was reported for first time from this plant. Furthermore the authors isolated and phytochemically investigated seven other clerodane diterpenoids and five alkaloids including liriodenine, (-)-anonaine, (+)-isoboldine, (-)-asimilobine and hordenine from the ethanolic extract of leaves of *P. longifolia*.

Table: 3.8. A list of phytochemicals from *Polyalthia longifolia* and their biological activities

Phytochemical components	Plant part	Biological activities	References
3α,5α,16R-trihydroxyhalima-13(14)-en-15,16-olide, 16β-hydroxycleroda- 3,13-dien-15,16-olide, 5-Hydroxy-6-methoxonychine, 6-Hydroxy-7-methoxyonychine, Stepholidine & Norboldine	Leaves	Cytotoxicity	Chen et al.,2000
Kolavenic acid & 16-oxo-cleroda-3,13(14)E-dien-15-oic acid	Seed	Antifungal & antibacterial	Islam et al., 2001
Pendulamine-A, Pendulamine-B & Penduline	Root	Antimicrobial	Faizi et al., 2003
(3S,4R)-3,4,5-trihydroxypentanoic acid-1,4-lactone	Stem	Antibacterial & antifungal	Faizi et al., 2003
16α-hydroxy-cleroda-3,13(14)-Z-diene-15,16-olide	Seed	Antibacterial & antifungal	Marthanda et al., 2005
	Leaves	Antileishmanial	Misra et al., 2010
16-hydroxycleroda-3,13(14)E-dien-15-oic acid	Bark	Anti-inflammatory	Chang et al., 2008
16(*R* and *S*)-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide, 16 (*R* and *S*)-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide-2-one, (4→2)-*abeo*-16(*R* and *S*)-hydroxy-cleroda-2,13(14)*Z*-dien-15,16-olide-3-al & 3β ,16α-dihydroxy-cleroda-4(18), 13(14)*Z*-dien-15,16- olide	Leaves, Stem, Green berries, Flowers, Root & bark	Antimicrobial	Faizi et al., 2008
2', 4'-dihydroxy-3'-methoxychalcone	Stem bark	Cytotoxicity	Ghani et al., 2012

Contd….

(Contd…) A list of phytochemicals from *Polyalthia longifolia* and their biological activities

Phytochemical components	**Plant part**	**Biological activities**	**References**
(−)-3α,16α-dihydroxycleroda-4(18),13(14)*Z*-dien-15,16-olide & (−)-3β, 16α-dihydroxycleroda-4(18), 13(14)*Z*-dien-15,16-olide.	Leaves	Inhibitory activity on four cancer cell lines.	Sashidhara et al., 2009
6α,16-dihydroxycleroda-3,13-dien-15-oic acid, 6α,16-dihydroxycleroda 4(18),13-dien-15-oic acid,	Stem	Anticancer	Lee et al., 2009
4α,18*β*-epoxy-16-hydroxyclerod-13-en-15-oic acid,			
(-)-8-oxo-10-hydroxy-2,3,9 trimethoxyberberine,			
(-)-8-oxo-2,11-dihydroxy-3,10-dimethoxyberberine,			
(-)-8-oxo-11-hydroxy-2,3,9,10-tetramethoxyberberine & (-)-8-oxo-2,10-dihydroxy-3,9,11-trimethoxyberberine			
(-)-14,15-bisnor-3,11E-kolavadien-13-one,	Leaves	Antimicrobial	Sashidhara et al.,2009
(-)-16-oxocleroda-3,13(14)E-dien-15-oic acid,			
(-)-16α-hydroxycleroda-3,13(14)Z-dien-15,16-olide**,** (+)-(4-->2)abeo-16(R/S)-2,13Z-kolavadien-15,16-olide-3-al,			
(-)-3β,16β-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide,			
(-)-3,12E-kolavadien-15-oic acid-16-al & (-)-labd-13E-en-8-ol-15-oic acid			
24-methylenecycloartane-3β, 16β, 23b-triol, longitriol & longimide A and longimide B	Leaves	Cytotoxicity against cancer cell lines.	Sashidhara et al., 2010
Flavonoids viz., rutin, chrysin and daidzein	Leaves	Antibacterial, antiradical & cytotoxicity	Sampath and Vasanthi, 2013

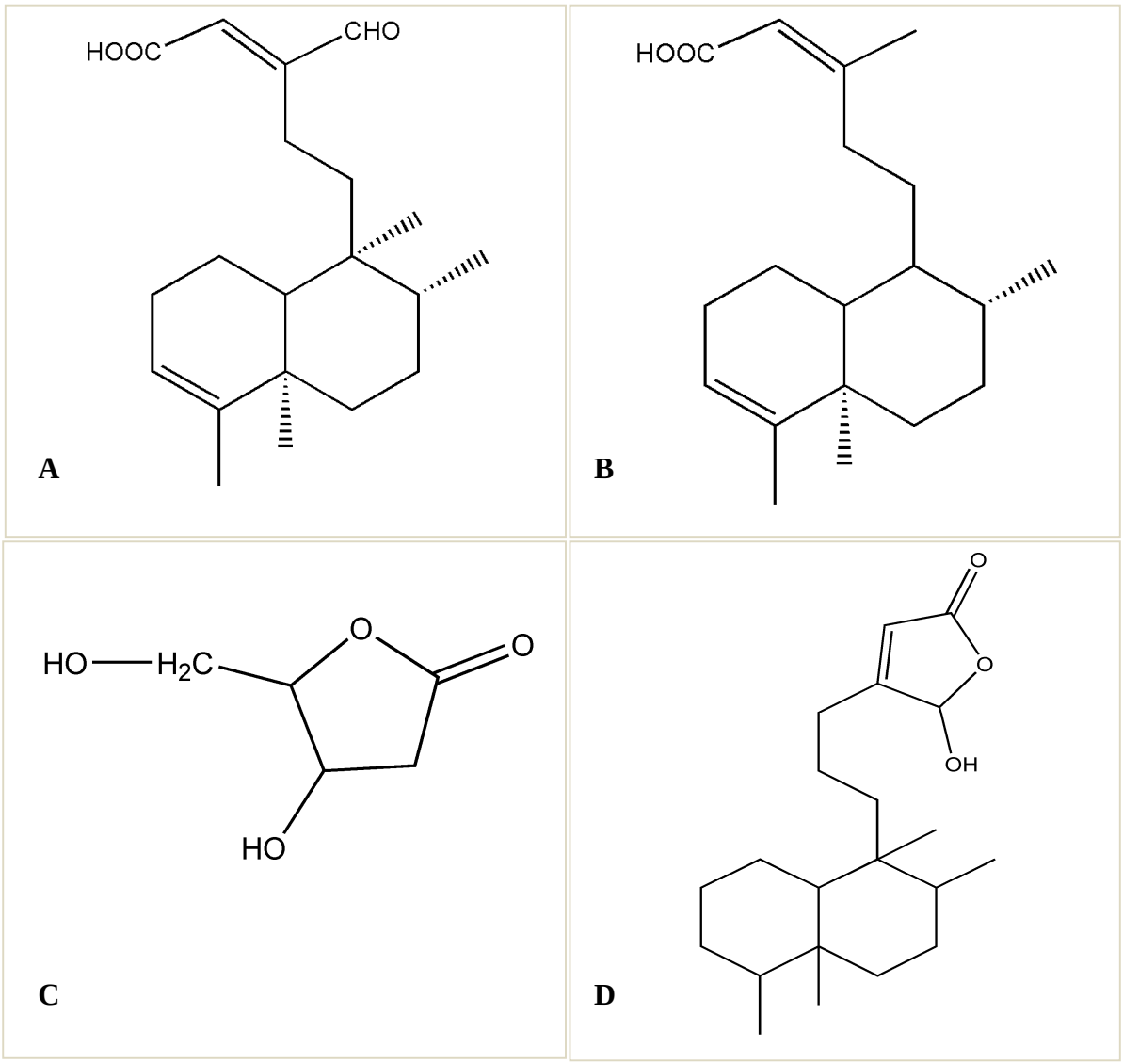

Fig. 3.4. Structure of antifungal phytochemical compounds from *Polyalthia longifolia*

A: 16-oxocleroda-3,13(14)*E*-dien-15-oic acid

B: Kolavenic acid (Islam et al., 2001)

C: (3S,4R)-3,4,5-trihydroxypentanoic acid-1,4-lactone (Faizi et al., 2003)

D: 16α-hydroxy-cleroda-3,13(14)-Z-diene-15,16-olide (Marthanda et al., 2005)

3.2. Materials and methods

3.2.1. Selection and collection of plant materials

The plant materials were selected on the basis of literature reports of their traditional ethnomedicinal uses and bioactivity as well as availability in **s**ub-Himalayan region of West Bengal. The particular plant parts can be selected depending on where the metabolites of interest accumulated. Altogether 14 plant species (Figs. 3.5-3.6) were studied for their biological activity and phytochemical analysis. Table 3.9 summarizes the areas from which the plant species were collected. Most of the plant materials were collected from local areas within and outside campus of University of North Bengal. Some of the plants were collected from forest areas of Cooch Behar and Sukna located in the Terai region of the eastern Himalayas and also from Terai region of Rajgir hills in Bihar. Fresh disease free leaves of different plant species were collected. The effective plant species were identified by Prof. A.P. Das of the Department of Botany, University of North Bengal. Voucher specimen of each species was deposited in the herbarium of Department of Botany, University of North Bengal.

3.2.2. Test organisms

3.2.2.1. Fungal pathogens of tea

Altogether five fungal pathogens which severely affect the tea plantation in North-Eastern region of India were used as test pathogens in the current study (Table 3.10). These cultures, viz. *Colletotrichum camelliae*, *Curvularia eragrostidis*, *Lasiodiplodia theobromae*, *Pestalotiopsis theae* and *Rhizoctonia solani* were kindly gifted by Dr. Aniruddha Saha, Molecular Plant Pathology and Fungal Biotechnology laboratory, Dept. of Botany, University of North Bengal, Siliguri, India (Saha et al., 2005a; Mandal et al., 2006; Saha et al., 2008). The identity of the pathogens *C. eragrostidis*, *L. theobromae* and *R. solani* were further confirmed by Indian Agricultural Research Institute (IARI), New Delhi and identification numbers were assigned to them.

Table: 3.9. Collection sites of plants used in the present study

Name of Plants	Family	Area of collection	Nature of location
Bidens pilosa Linn.	Asteraceae	N.B.U. campus	Waste area
Clausena excavata Burm.f.	Rutaceae	N.B.U. campus	River side
Datura stramonium Linn.	Solanaceae	Urban area	Road side
Datura innoxia Mill.	Solanaceae	N.B.U. campus	Road side
Emblica officinalis L.	Euphorbiaceae	N.B.U. campus	Garden area
Eucalyptus globulus	Myrtaceae	N.B.U. campus	Road side
Lantana camara L.	Verbenaceae	Sukana, Darjeeling	Waste area
Leonurus sibiricus Linn.	Lamiaceae	Cooch behar	Forest area
Maesa indica	Myrsinaceae	Cooch behar	Forest area
Ocimum sanctum	Lamiaceae	Terai region of Rajgir hills (Bihar)	Near cultivated area
Polyalthia longifolia var *pendula*	Annonaceae	N.B.U. campus	Garden
Piper betle Linn.	Piperaceae	Village area	Road side
Syzygium cumini	Myrtaceae	N.B.U. campus	Road side
Xanthium strumarium L.	Asteraceae	Urban area	Road side

Table: 3.10. Major fungal pathogens of North-East India affecting tea plantations which were used in the present study

Fungal Culture	Strain identity	Disease produced	Plant part affected
Pestalotiopsis theae	PT01	Grey blight	Leaf
Colletotrichum camelliae	CC01	Brown blight	Leaf
Lasiodiplodia theobromae	ITCC 5446.02	Root rot or Diplodia disease	Root, stem & leaf
Curvularia eragrostidis	ITCC 4150.2K	Leaf spot	Leaf
Rhizoctonia solani	ITCC 5995.05	Root rot	Root

3.2.2.2. Fungal pathogens of crop plants other than tea

The antifungal activity of the extracts was assessed against the plant pathogenic fungal strains, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Fusarium equiseti*, *Macrophomina phaeseolina*, *Sclerotium rolfsii, Rhizopus stolonifer* and *Sclerotinia sclerotiorum* (Table 3.11). The ITCC strains were either obtained directly from the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi or they were isolated as plant pathogens from crop fields and their identities were authenticated by IARI, New Delhi.

Table: 3.11. Pathogens of crop plants used in this study

Name of pathogens	Identity (ITCC No.)*	Crops affected	Nature of disease
Colletotricum gloeosporioides	5446.02	Brinjal, mango, tomato wide host range	Anthracnose in fruits & vegetables
Fusarium equiseti	6566.07	Soyabean, cumin, brinjal	Rot in stem base & root
Fusarium oxysporum	6246	Wide host range	Vascular wilt
Macrophomina phaeseolina	5519	Wide host range	Damping off, seedling blight, collar rot, stem rot, charcoal rot & root rot
Rhizopus stolonifer	6283	Wide host range	Soft rots in post harvest disease in fruits & vegetables
Sclerotium rolfsii	6415	Wide host range	Root rot, stem rot, wilt & foot rot
Sclerotinia sclerotiorum	6094	Wide host range	Serious cottony rot, watery soft rot, stem or fruit rot, post harvest fruit & vegetable decay

* Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi

3.2.2.3. Fungal pathogens of clinical importance

Some important fungal pathogens of humans were included as test organisms in the present study. The fungal strains, *Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans*, *Candida tropicalis*, *Cryptococcus curvatus*, *Filobasidiella neoformans*, *Mucor circinelloides* and *Trichosporon cutaneum* (Table 3.12) were used for assessing the antifungal activity of the plant extracts. All the strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Table: 3.12. List of fungal pathogens of clinical importance used during this study

Fungal culture	Strain Identity*	Diseases produced
Aspergillus niger	MTCC 2425	Lung disease-Aspergillosis, hearing problems & even hearing loss.
Aspergillus fumigatus	MTCC 6594	Chronic pulmonary infections, allergic bronchopulmonary Aspergillosis in immunocompromised individuals.
Candida albicans	MTCC 183	Opportunistic oral and genital infections in humans, Candidal onchomycosis & candidiasis in immunocompromised patients.
Candida tropicalis	MTCC 184	Candidaemia, invasive candidiasis, skin and nail scraping.
Cryptococcus curvatus	MTCC 2698	Pulmonary Cryptococcosis & basal meningitis.
Filobasidiella neoformans	MTCC 1431	Cause systemic infection, including fatal meningitis (meningoencephalitis), diabetic (The infection from *F. neoformans* in the brain can be fatal if untreated).
Trichosporon cutaneum	MTCC 1963	Superficial skin disease onychomycosis, otomycosis and hypersensitivity pneumonitis.
Mucor circinelloides	MTCC 3944	Skin disorder dermatitis or pulmonary fungal infection. Diarrhea, zygomycosis or mucormycosis and severe tissue necrosis that requires surgical debridement.

*MTCC = Microbial type culture collection, Chandigarh

3.2.3. Soxhlet extraction

Fresh leaves were thoroughly washed with distilled water and shade dried at room temperature for five to ten days. The dried leaves (120gm) were ground to moderately fine powder (1mm) and extracted in a soxhlet apparatus using appropriate solvents (600ml), for 15 hours at 35-45^{o}C. The extract was concentrated to dryness under vacuum in a rotary evaporator (Eyela, Japan). The residue (15gm) obtained as a gummy solid mass was dissolved in proper solvent and used for bioassay.

3.2.4. Bioassay methods

3.2.4.1. Preparation of spore and mycelial suspension

For preparing spore suspension, potato dextrose agar plates (9 cm) were inoculated with the mycelial discs of the fungal pathogens individually in separate plates and incubated for 10-12 days at 28ºC for adequate sporulation. Spore suspension was prepared either in sterile distilled water or in sterile Richard's medium and about 5-10 ml was added aseptically to the fungal cultures. The surface of the mycelial mat on the flooded plates was brushed gently with inoculation needle to loosen the spores. The resulting suspension was filtered through cheese cloth to remove mycelial fragments. For preparing mycelial suspension, the mycelium was scrapped lightly in sterile distilled water with inoculation needle and filtered through sterile double-layered muslin cloth in order to avoid any traces of agar. The concentration of spores in the filtrate was measured using haemocytometer and the final concentration was adjusted to 1 x 10^{6} spores ml^{-1} (Saha et al., 2005a).

3.2.4.2. Agar cup bioassay

The fourteen different plant extracts (Table 3.13) were screened for antifungal activity by agar cup diffusion method. Potato dextrose agar (PDA) medium was autoclaved at 121^{o}C for 15 min, cooled to 45^{o}C and 1 ml of pure spore suspension or mycelia suspension of the test pathogen was mixed with 19 ml of molten

medium and poured into sterile petriplates of 9 cm diameter. Agar cups were prepared with sterile cork-borer (4 mm diameter) in the PDA plates after solidification of the medium seeded with spores of the test fungi. Plant extracts (50µl) were introduced into each well and the plates were incubated for 48-72 hours at 28^{o}C. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth around the plant extracts. Complete antifungal assay was carried out under strict aseptic conditions. The zones of inhibition were measured with scale in mm and the experiment was carried out in triplicate.

3.2.4.3. Bioautography

Antifungal activities of the crude extract and the partially purified column fractions and the purified compounds were tested by bioautography following the method of Saha et al. (2005b). Glass-backed silica gel TLC plates (10 cm x 20 cm) were prepared manually by coating with silica gel G (for TLC) (SRL, India). The plates were air-dried for 2-3 days and each plate was activated by heating at 70^{o}C for 45 minutes prior to sample-loading. Concentrated test extracts were spotted on the activated TLC plates and developed either in hexane : ethyl acetate: methanol (60:40:1) or in hexane : ethyl acetate (70:30 or 80:20 v/v) as solvent. The developed chromatograms were air-dried until the solvent evaporated completely. Subsequently, spore suspension (10^6 spores/ml) prepared from the test fungi was mixed with Richard's medium (15g/L) and sprayed with an atomizer on dried TLC plates. The plates were incubated in a humid chamber at 28^{o}C for 2-5 days. Inhibition zones, which appeared as clear white spots on a background of thick mycelial growth, indicated the presence of antifungal compounds. R_f value of the inhibition zone was noted. The centre of inhibition zones where the four zone diameters meet (diameters at 45^{o} angle to each other) was used for calculating R_f.

3.2.4.4. Bioautography against *Candida albicans*

Isolated compounds were spotted on precoated silica gel 60 F254 aluminium TLC plates (Merck) for the confirmation of antifungal activity. The compounds were separated on TLC plates as stated above and the plates were dried to overcome the effect of solvents. Chromatograms were placed in sterile Petri plates. Molten PDA medium was mixed with phenol red (0.02%) and an inoculum of cell suspension of *Candida albicans* at tolerable temperature. The mixture was poured evenly over developed TLC plates and covered with lid. The plates were incubated at 28^{o}C for 24 hrs. (Lopez et al., 2007). Antifungal activity was detected by formation of clear inhibition zones on the chromatograms which was visible against the red background of fungal growth.

3.2.4.5. Minimum inhibitory concentration (MIC) by microdilution bioassay

Antifungal activity of the isolated compound was tested against a wide range of fungal strains by using the 96-well micro-titre plate assay. To determine the MIC, the active constituent was serially double diluted in ethanol (2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 and 0.019 mg/ml) and 50 µl of each of the different concentrations were pipetted into the wells of the micro-titre plate. The standard fungicide bavistin and the antifungal antibiotic nystatin were included for comparison. Next, ethanol was allowed to evaporate and then, each well was loaded with a mixture (100 µl) of sterile PDB and fungal inoculum. The inoculum was prepared from 4-8 day old fungal cultures in the form of either spore suspension (10^6 spores ml^{-1}) or fine mycelia fragment in PDB as described above (Section **3.2.4.1**). Positive control wells contained inoculum and PDB only, without any test compound. A negative control set contained the test compound and PDB only. The plates were covered with plastic lid and incubated in a growth chamber at 28^{o}C. Fungal growth was monitored after 48 h by measuring absorbance at 600 nm using a microtitre-plate reader (Mios Junior, Merck). A zero hour reading was taken as blank. The MIC was considered as the lowest final concentration that did not record any growth.

3.2.4.6. Determination of reducing Power

Reducing power of phytochemical compounds was tested by the method of Wu et al. (2011) with some modification. An aliquot of 0.5 ml of extract containing different concentrations of the test compounds (19.5, 39.0, 78.0, 156, 312 and 625 µg ml^{-1}) was mixed with 0.5 ml of phosphate buffer (0.2M, pH 6.6) and 0.5 ml of potassium ferricyanide (1%). Ascorbic acid was used as positive control. The mixed solution was incubated at 50°C for 20 minutes. After incubation, 0.5 ml of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. From the upper layer, 0.5 ml of solution was removed, mixed with 0.5 ml of distilled water and 0.1 ml of $FeCl_3$ (0.1%) and the absorbace at 700 nm was noted. An increase in the absorbance indicated reducing power.

3.2.4.7. DPPH free radical scavenging activity

Free radical scavenging activity of nordentatin, dentatin, clausenidin and osthol were measured using 1,1-diphenyl-2-picryl-hydrazil (DPPH) by the method of Patel and Patel (2011). In brief, 1ml methanol solution of different concentrations (19.5, 39, 78, 156, 312 and 625 µgml^{-1}) of test compounds were taken in test tubes. To each of the tubes, 0.5 ml of 0.2 mM DPPH-methanol solution was added and the volume was adjusted to 2 ml by methanol. Negative control consisted of DPPH solution without test compounds while ascorbic acid solution was used as positive control. The mixed solution was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm by spectrophotometer (Systronics, Visiscan 167). The percentage inhibition was calculated from the following equation: % inhibition= [(absorbance of control-absorbance of tested sample)/absorbance of control] X100.

3.2.4.8. DPPH free radicals scavenging activity on TLC plate autographic assay

The antioxidant activity was detected on TLC plates by the method of Kannan et al. (2010) with some modification. The TLC plates (Merck, UV 254 nm) were

activated at 70^{o}C for 30 minutes. The compounds were spotted on TLC plates and separated in petroleum ether-ethyl acetate (70:30) solvent. The developed TLC chromatograms were dried and observed under UV light (254 nm and 365 nm). The separated UV active spots were marked on TLC plates and subsequently sprayed with 0.2 mM DPPH solution. The plates were incubated in dark for 20 minutes at room temperature. Appearance of yellow spot in purple background indicated positive test.

3.2.4.9. Oxidative DNA damage protective activity

Oxidative DNA damage protective activity was tested on pUC18 super coiled plasmid DNA. Super coiled plasmid DNA was isolated by Genei Pure plasmid purification kit (Merck, India). The DNA protective assay was performed using protocol of Kumar and Chattopadhyay (2007) with some modification. In brief, the experiments were performed in 15 µl volume in microcentrifuge tubes containing super coiled pUC18 plasmid DNA (5µl, containing about 300-400 ng DNA) in TE buffer (pH 8.0) and test compounds (5µl); the final volume was adjusted with TE buffer. This solution was incubated for 10 min at room temperature and H_2O_2 was added at a final concentration of 20 mM. Apart from the experimental set, three other sets were included: a control set containing all components (plasmid and H_2O_2) except test compounds, a positive control using quercetin instead of the test compound and a third blank set containing only super coiled plasmid DNA. The reaction was initiated by UV irradiation under the surface of UV transilluminator (Bangalore Genei PVT Ltd., Bangalore, India) with intensity of 6x8W-312 nm and continued for 5 min. The irradiated reaction mixtures were mixed with gel loading buffer and immediately loaded in the wells of 1% agarose gel containing ethidium bromide and electrophoresis was performed at 55V for 2 hours. Following electrophoresis the gel was viewed under UV in a UV transilluminator (Bangalore Genei PVT Ltd., Bangalore, India) and photographed.

3.2.5. General experimental procedure

Purification was done by silica gel (60-120 mesh SRL, India) for column chromatography and occasionally in later stages with sephadex LH-20 (Sigma) column chromatography. Precoated silica gel 60 F254 aluminium sheets (Merck, India) was used for TLC unless stated otherwise. Silica gel G and silica gel GF 254 with binder (SRL, India) were used for bioautography. Melting point was determined in open capillary tube method and remains uncorrected. The UV spectrum was recorded in UV1/160A UV-visible recording spectrophotometer, (Shimadzu, Japan) (λmax in nm). The FT-IR spectrum was detected in KBr and nujal in a Shimadzu-8300 FT-IR spectrophotometer (ν_{max} in cm^{-1}). ESI-MS spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer (SAIF, Central Drug Research Institute, Lucknow). The NMR 1D (^{1}H- and ^{13}C-) and 2D spectra were detected in $CDCl_3$, (300MHz) in a Bruker-AV 300 spectrometer with TMS as internal reference**.** The chemical shifts of NMR spectrophotometer are in δ ppm and *J* in Hz.

3.2.6. Preliminary characterization of phytochemical components

The crude extracts of plant samples were analyzed phytochemically by TLC using ultraviolet light and spray reagents. The crude and purified extracts were dissolved in proper solvent at 10mg/ml concentration. About 10µl (100 µg) of extracts were spotted on activated (70^{o}C for 45 min) precoated silica gel 60 F254 aluminum TLC plates (Merck). After spotting, the plates were developed with hexane : ethyl acetate : methanol (60:40:1v/v) or hexane : ethyl acetate (60:40 / 70:30/ 80:20). The developed chromatograms were viewed under UV light (254nm and 365nm wavelengths). The spray reagents vanillin–sulphuric acid, anisaldehyde–sulphuric acid, iodine reagent and folin ciocalteu reagent were prepared in laboratory (Appendix-B) by standard protocol (Wagner and Bladt, 1996; Harborne, 1998) and sprayed on the developed chromatograms with an atomizer. The colours of the spots were noted and the R_f was calculated. The result of chemical analysis was determined based on colour of the spots as given

in table 3.13. After that, the plates were developed with hexane : ethyl acetate : methanol (60:40:1v/v), hexane : ethyl acetate (60:40, 80:20, and 90:10).

Table: 3.13. Chemical analysis of phytochemicals separated on TLC plates on the basis of colour reactions of different compounds (Wagner and Bladt, 1996)

Treatments	Phytochemicals	Colour appeared
UV light exposure		
UV254nm	Coumarins	Blackish Blue
UV365nm	Coumarins	Blue
Spray reagents		
Vanillin-sulphuric acid (VS)	Monoterpene alcohol Bitter principle Saponin	Blue/ Blue-green
Anisaldehyde-sulphuric acid (AS)	Triterpene	Red-violet
	Terpenes	Violet
	Essential oil	Green
	Bitter principle	Dark green
Folin Ciocalteu's	Phenolic	Blue
Iodine	Unsaturated	Yellow/ Yellow brown

3.2.7. Extraction and Purification of phytochemicals from leaf extracts of *Clausena excavata*

3.2.7.1. Compound E-1

The dried and powdered leaves of *C. excavata* (200g) were extracted with dichloromethane in a soxhlet apparatus for 20 hours at 35^{o}C. The extract was concentrated under reduced pressure to give a brown sticky solid (15g) which was subjected to silica gel column chromatography. Elution with n-hexane, n-hexane : dichloromethane (3:1, 1:1, 1:3), dichloromethane, dichloromethane : ethyl acetate (3:1, 1:1, 1:3), ethyl acetate, ethyl acetate : methanol (3:1, 1:1, 1:3) and methanol gave thirteen fractions (F_1 to F_{13}). Based on activity tested through bioautography on TLC plates, fraction F_7 (2g) was rechromatographed on a silica gel column, eluting with petroleum ether (200ml) and EtoAc : hexane (2%, 5%, 10%, 15%, 20%, 25%, 30% and 35%) by which a total of 9 fractions (F_1 to

F_9) were collected. The bioactive fraction F_7 and F_8 was fractionated into 40 sub-fractions (SF_1 to SF_{40}). Six sub-fractions (SF_{30}-SF_{35}) were pooled and crystallized with hexane-ethyl acetate to give **E-1**(110mg). The crystallized compound was tested for antifungal activity by bioautography.

3.2.7.2. Compound E-2

Dried and powdered leaves of *C. excavata* (250g) was extracted with ethyl acetate (700ml) in Soxhlet apparatus for 18 hours at 35^{o}C. The extract was concentrated under reduced pressure to obtained 18g of brown solid mass. This crude extract was subjected to silica gel column chromatography using gradient elution with increasing polarity. Elution with petroleum ether and petroleum ether : ethylacetate (98:2, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55. 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95 and 0:100) gave 22 fractions (F_1 to F_{22}) of 200ml each. All fractions were concentrated under vacuum, monitored by TLC (developed with hexane : ethyl acetate (60:40) under UV light and bioassayed following bioautography method. Based on antifungal activity and TLC band pattern, fractions F_8 and F_9 were combined and rechromatographed on silica gel column eluting with hexane and gradient mixture of hexane and ethylacetate with increasing polarity (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45 and 50:50) to obtain 11 fractions (200 ml each). The sub fractions SF_7 and SF_8 were crystallized with hexane-ethylacetate to obtain **E-1** (300mg) and **E-2** (80mg) respectively. The crystallized compounds were finally monitored on TLC, bioassayed by bioautography and further analyzed by UV-Vis, IR, NMR (^{1}H- and ^{13}C-) and ESI-MS spectroscopy.

3.2.8. Extraction and purification of phytochemicals from *Clausena excavata* root bark extracts

The collected root bark was thoroughly washed with water, chopped into small pieces and shade dried at room temperature for seven to ten days. The dried

bark was ground to moderately fine powder (2-5mm) and the powdered bark (250g) was extracted with acetone (700ml) in soxhlet apparatus for 36 h at 40^{o}C temperature. The solvent was evaporated under reduced pressure to obtain brown sticky mass (16gm). This crude extract was dissolved in chloroform (30ml), mixed and dried over silica gel (60-120 mesh size, 30gm) and was subjected to silica gel column chromatography. Elution with petroleum ether (300ml) and petroleum ether : ethylacetate (95:5, 90:10, 85:15, 80:20, 75:25 (sub-fraction a & b), 70:30, 65:35, 60:40, 55:45, 50:50, 45:55 and 40:60) yielded a total of fourteen fractions (F_1-F_{14}) of 300ml each. Each fraction was vacuum-concentrated and monitored by TLC (developed with hexane : ethyl acetate : methanol (60:40:1) or hexane : ethyl acetate (60:40, 80:20, and 90:10) under UV light and by chromogenic sprays (Anisaldehyde–sulphuric acid) and also bioassayed following bioautography method. The fraction F_6 (petroleum ether : ethylacetate as 75:25) was sub fractionated into SF_{6a} and SF_{6b}. On overnight standing, fraction F_5 (petroleum ether : ethylacetate as 80:20) yielded white (1.5g) and sub-fraction SF_{6b} yielded yellow crystal granules (1.2g).

Crystallized compounds were removed from solvent and then recrystalized with petroleum ether and ethylacetate to obtain pure compounds (**CR-1** (250mg) and **CR-2** (140mg). The remaining part of fraction F_5, sub-fraction SF_{6b} and SF_{6a} were combined. The combined fractions were rechromatographed on silica gel column and six sub-fractions (SSF_1-SSF_6) were eluted with petroleum ether : ethyl acetate (98:2, 95:5, 90:10, 85:15, 80:20 and 75:25). Fractions SSF_4, SSF_5 and SSF_6 were crystallized with petroleum ether and ethyl acetate to obtain pure compounds **CR-4** (40mg), **CR-1** (80mg) and **CR-2** (60mg) respectively. Fraction SSF_3 was rechromatographed on silica gel (100-200 mesh SRL, India) column and eluted with hexane (F_1), hexane : ethyl acetate (98:2-F_2, 95:5-F_3, 93:7-F_4, 90:10-F_5 and 85:15-F_6) to yield six major fractions of 200 ml each. The fration eluted with 90:10 –hexane : ethyl acetate was sub-fractionated into 20 parts, each 10 ml. Fraction F_4 and F_5 were crystallized using hexane-ethyl acetate to obtained

compounds **CR-5** and **CR-3**. The purified compounds were further analysed by spectroscopic methods to elucidate their structures.

3.2.9. Extraction and purification of antifungal compounds from *Xanthium strumarium*

Collected *X. strumarium* leaves were washed with tap water and dried at room temperature for 5-7 days. Dried leaves were ground to fine powder (300g) and extracted with dichloromethane in soxhlet apparatus at 37^{o}C for 20 hours. The solvent was evaporated under vacuum to obtain crude sticky solid mass (20gm). The crude extract was dissolved in chloroform (50ml) and 5% aqueous lead acetate solution was added and mixed by shacking. The organic layer was separated by separating funnel, washed with 1% aqueous NaCl solution, dried over Na_2SO_4 and made to fine powder. The crude extract was subjected to silica gel column chromatography eluting with petroleum ether and ethyl acetate : petroleum ether (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50%) to produce 11 fractions (F_1-F_{11}), each 300ml. All fractions were concentrated under vacuum and tested for antifungal activity by bioautography method and monitored by TLC which was developed using hexane : ethyl acetate (60:40) and sprayed with vanillin–sulphuric acid, anisaldehyde–sulphuric acid and Iodine vapor or viewed under UV light. Based on biassay results, fractions F_7 and F_8 were combined and rechromatographed on aluminium oxide (neutral) column using gradient elution with increasing polarity with petroleum ether (200ml) and ethyl acetate : hexane (10%, 15%, 20%, 25%, 30% and 35%). Total 7 fractions (SF_1-SF_7) were collected, SF_5 and SF_6 were combined and rechromatographed on LH-20 column (Sigma). Elution with ethylacetate : petroleum ether (10%, 15%, 20%, 25% and 30%) yielded 5 fractions. Of these, two fractions (25% and 30% ethylacetate : pet-ether) were finally purified by crystallization under 4^{o}C in refrigerator to obtained **X-1** (70mg). The fractions F_9, F_{10} and F_{11} were combined and rechromatographed on silica gel column eluting with ethylacetate : petroleum ether (10%, 15%, 20%, 25%, 30%, 35%, 40% and 45%) to obtain 9

fractions (200 ml each). All fractions were concentrated and the fraction eluted with 40% ethyl acetate : petroleum ether was found to be bioactive. The purified **X-2** (75mg) was obtained from this fraction as a semisolid sticky mass. The antifungal compounds were further studied by spectroscopic methods for elucidation of the respective structures.

3.2.10. Extraction and purification of antifungal compound from *Piper betle* leaves

P. betle leaves were collected from cultivation sites and washed with tap water and shade dried at room temperature for 5 days. The dried leaves were powdered (250gm), packed into soxhlet column and extracted with dichloromethane for 20 hours at 35^{o}C. The solvent was evaporated under reduced pressure at 35^{o}C to obtain a gummy semi solid mass (14gm) as crude extract. The crude extract was dissolved in chloroform (100ml) and thoroughly mixed with 5% aqueous solution of lead acetate. The fats were precipitated in aqueous lead acetate upper layer and lower organic layer was separated by separating funnel. This step was repeated twice. Organic layer containing bioactive compounds was washed with 1% aqueous NaCl solution, dried over Na_2SO_4, prepared to form a fine powder and finally packed onto silica gel column. Elution with petroleum ether and then with ethyl acetate : petroleum ether (5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60 and 50:50) gave ten fractions (F_1-$F_{10,}$ 300ml each). Each fraction was vacuum-concentrated and monitored by TLC (developed with hexane : ethyl acetate (70:30) under UV light and by chromogenic sprays, Folin & Ciocalteu's reagent and Iodine vapour) and also bioassayed following bioautography method. Fraction 5 (2g) was rechromatographed using ethyl acetate - hexane (5:95, (10:90), (15:85) and (20:80) to yield four fractions (SF_1-SF_4) of 300 ml each. Fraction SF_2 was sub-fractionated into six sub-fraction (SF_{2a}, SF_{2b}, SF_{2c}, SF_{2d}, SF_{2e} & SF_{2f}). Each fraction was vacuum-concentrated and monitored by TLC (developed with hexane : ethyl acetate (70:30) under UV light and by chromogenic sprays, Folin & Ciocalteu's

reagent and Iodine vapour) and also bioassayed following bioautography method. Sub-fractions (SF_{2c}-SF_{2f}) were combined and further rechromatographed, eluting with chloroform and methanol : chloroform (2%, 5% and 10%). The 1st chloroform fraction was sub-fractionated (SF_1, SF_2, and SF_3). Sub-fraction SF_3 was obtained as yellowish oil as a pure compound (**PB-1**). The antifungal compound was further studied by spectroscopic methods to describe the structure.

3.2.11. Extraction and purification of antifungal compound from *Ocimum sanctum* leaves

Ocimum sanctum leaves were collected from cultivated field areas of Rajgir, Nalanda, Bihar. The leaves were thoroughly washed with tap water and shade dried at room temperature for 5 days. The dried leaves were ground to make fine powder (200gm) and extracted with soxhlet in dichloromethane for 22 hours at 35°C. Solvent was evaporated under reduced pressure at 35°C temperature to obtain gummy semi solid mass (10gm) as crude extract. The crude extract was dissolved in chloroform (50ml), dried over Na_2SO_4 and prepared as fine powder, which was then packed onto silica gel column. The fractions were eluted using petroleum ether and then polarity was increased with ethyl acetate : petroleum ether (5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60 and 50:50) to produce ten fractions (F_1-F_{10}, 300ml each). Fractions were tested for antifungal activity by bioautography and monitored by TLC under UV. The bioactive fraction (F_3, 1.1gm viscous) was rechromatographed and eluted with hexane, ethyl acetate : hexane (2:98), (5:95), (7:93), (10:90) and (15:85%). Total six fractions (SF_1-SF_6) were obtained of which SF_3 and SF_4 were combined based on bioactivity and rechromatographed on silica gel (100-200 mesh size) column. The column was eluted with hexane, ethyl acetate : hexane (2:98, 5:95, 7:93, 10:90 and 12:88) and again 6 fractions (SSF_1-SSF_6) were collected. Fractions SSF_3 and SSF_4 were dried under reduced pressure to obtained purified compound (**OS-1)** as yellowish oil mass (65mg) which was further studied by spectroscopic methods and the structure was determined.

3.2.12. Extraction and purification of antifungal compound from *Polyalthia longifolia*

The leaves of *P. longifolia* was collected and washed with tap water and shade dried at room temperature for 5 days. The dried leaves were powdered (300gm), packed into soxhlet column and exhaustively extracted with dichloromethane for 20 h at 35^{o}C. The extract was concentrated under reduced pressure to give a gummy semi solid mass (15gm) which was dissolved in chloroform. Aqueous lead acetate solution (5%) was added and well shaken to precipitate the fats which were obtained in aqueous layer and separated out using a separating funnel. The chloroform layer containing bioactive compounds was washed with 1% aqueous NaCl solution, dried over Na_2SO_4, powdered and finally loaded onto silica gel column. The extract was eluted by gradient solution with increasing polarity. Elution with petroleum ether and ethyl acetate : petroleum ether (5:95, 10:90, 15:85, 20:80, 25:75, 30:70 and 35:65) gave eight fractions (F_1-F_8). Each fraction was concentrated under vacuum and bioassayed following bioautography method and monitored by TLC using hexane : ethyl acetate (80:20v/v). The developed chromatograms were seen under UV and monitored by spray reagent (anisaldehyde–sulphuric acid solution). The fraction F_6 and F_7 which showed antifungal activity were combined and rechromatographed on silica gel column using ethyl acetate: Pet-ether again as the eluting solvent to yield four sub fractions (SF_1-SF_4). Sub-fraction SF_2 (10% EtOAc : Pet-ether) which was obtained as a yellow semisolid mass (450mg) and tested positive in bioautography test was further purified by silica gel column with 10% EtOAc : Pet-ether and four fractions ($SF_{2.1}$-$SF_{2.4}$) were collected, concentrated and bioassayed. The bioactive fractions ($SF_{2.2}$ and $SF_{2.3}$) were combined (340mg) and subjected to sephadex LH-20 column chromatography using petroleum ether as the eluent to finally yield a bioactive colorless semisolid gum **PL-1** (300mg) which was analyzed by UV-Vis, IR and NMR (^{1}H- and ^{13}C-) spectroscopy.

3.2.13. Biotransformation of bioactive compound 16-oxocleroda-3, 13(14) E-dien-15-oic acid isolated from *Polyalthia longifolia*

3.2.13.1. Culture conditions and screening for biotransformation

Biotransformation experiments were performed using medium containing 10g glucose, 5g peptone, 5g KH_2PO_4, 5g yeast extract, 10ml glycerol and 5g NaCl in every 1000 ml distilled water. Round agar blocks (4 mm) containing fungal mycelia were excised from advancing zones of 4-6 d old fungal cultures in potato dextrose agar plates and used to inoculate 50ml of sterile medium taken in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 28^{o}C with shaking at 150 rpm for 24 h to produce stage I cultures. For biotransformation in stage II cultures, the grown suspension culture (1-5 ml) was used to inoculate fresh sterile medium (50 ml) amended with the test compound (**PL-1**) at sub-lethal concentrations, which was determined considering the MIC value obtained against the fungus. For *R. stolonifer* cultures, a final concentration of 0.06 mg ml^{-1} was maintained. Control sets consisted of fungal culture under identical conditions but without the substrate. Uninoculated sterile media containing the substrate at same concentration served as substrate control. Experiments were performed in triplicate and all sets of culture flasks were incubated at 28^{o}C on shaker for 5 days. During incubation, samples (5ml) were withdrawn from each flask at every 24 h interval and analyzed for the formation of new product. The aliquots from experimental and control sets were pooled separately and filtered to remove the fungal mycelia. The filtrate was extracted with chloroform and monitored by TLC analysis. *R. stolonifer* was identified after thorough screening with several fungi for the formation of a product having higher polarity and antifungal activity and used for preparative scale biotransformation experiments.

3.2.13.2. Preparative scale up of biotransformation of PL-1 by *R. stolonifer*

The liquid culture medium (4800ml) for stage II culture was equally divided into 32 flasks of 500 ml capacity, each containing 150ml medium. **PL-1** (288mg) was dissolved in 8ml of ethanol and equally distributed among 32 flasks containing

sterile biotransformation liquid medium. A 24 h grown stage I culture of *R. stolonifer* (10 ml) was used to inoculate each flask containing the substrate amended liquid medium and incubated for 4 d at 28^{o}C on a rotary shaker. Formation of the transformed product was monitored by TLC at 24 h intervals. After 4 d, the substrate was completely converted into the more polar product.

3.2.13.3. Extraction and purification of biotransformed product of clerodene diterpene (16-oxocleroda-3, 13(14) E-dien-15-oic acid)

After the completion of biotransformation, fungal mycelium was removed by filtering and subsequently all culture filtrates were combined together and extracted three times with $CHCl_3$ (4,500ml). The organic layer was separated and concentrated under reduced pressure to give yellowish sticky mass (650mg). The product was purified by silica gel column chromatography. Column was eluted first with petroleum ether and then serially with a gradient mixture of increasing polarity with pet ether : ethyl acetate (95:5, 90:10, 85:15, 80:20, 75:25, 70: 30, 65:35, 60:40, 55:45 and 50:50) which gave eleven fractions (F_1-F_{11}). TLC monitoring (including bioautography) of all fractions and subsequent concentration yielded **PL-2** (45 mg) obtained from the F_7 fraction which was analyzed by spectroscopic methods.

3.3. Results and Discussion

3.3.1. Bioactivity of the crude extracts

3.3.1.1. Agar diffusion bioassay

Agar cup bioassay showed that all fourteen plants were active against all the tested fungal pathogens (Table 3.14). The soxhlet leaf extracts of *Piper betle*, *Datura innoxia, Polyalthia longifolia* and *Clausena excavata* produced the largest inhibition zones against most pathogens (Fig. 3.7 – Fig. 3.9). Highest inhibition zone of 24 mm was recorded by *P. betle* against *C. camelliae* followed by 23 mm zone recorded by *P. longifolia* against the same pathogen. Other plants showing strong antifungal activity were *Xanthium strumarium* and *C. excavata*. Some plants showed wide variations in their antifungal activity, for example, *Syzium cumini* produced only 9 mm antifungal zone against *C. eragrostidis*. *Emblica officinalis* and *Lantana camara* were found to be least effective as it showed no inhibition zone against two fungi tested. The smallest inhibition zone was recorded as 8 mm produced by *E. officinalis* against *C. eragrostidis*. *C. camelliae* was found to be the most susceptible fungi among all the tested pathogens. *C. eragrostidis* on the other hand, was the least affected pathogen. Considering the overall performances of the plant species, five plants, *P. betle*, *O. sanctum*, *P. longifolia, C. excavata* and *X. strumarium* were selected for further phytochemical analysis.

3.3.1.2. Bioautography

Bioautography of soxhlet extracts prepared from *C. excavata, X. strumarium, P. longifolia, P. betle, O. sanctum* and *D. innoxia* leaves revealed antifungal activity against *C. eragrostidis, L. theobromeae* and *C. camelliae* (Table 3.15). The occurrence of antifungal components was evidenced by the presence of clear zones of inhibition on TLC plates. Benzene extract from *X. strumarium* and ethyl acetate extract from *C. excavata* showed two antifungal zones each while the rest of the three plant extracts produced single antifungal zones. The largest

inhibition zone of 3.8 cm was produced by *X. strumarium* and *P. betle* extracts against *C. camelliae. C. excavata* was also showed big inhibition zones (2.5 cm) against *C. camelliae* and *C. eragrostidis.* All these extracts were further chemically analysed by bioassay guided fractionation to purify the antifungal molecules and elucidate the structures.

Table: 3.14. Screening of antifungal potential of crude leaf-extracts of different plants against fungal pathogens of tea by agar diffusion bioassay

Name of plants	Plant part	Solvent for extraction	*Diameter of inhibition zone (mm)			
			***C.e.*	*L.t.*	*C.c.*	*P.t.*
Bidens pilosa	Leaf	Ethyl acetate	17	11	18	12
Clausena excavata	Leaf/ Root bark	Ethyl acetate	16	20	22	10
Datura stramonium	Leaf	Benzene	10	13	15	11
Datura innoxia	Leaf	Ethanol	14	12	18	12
Emblica officinalis	Leaf	Ethyl acetate	8	NA	13	NA
Eucalyptus globulus	Leaf	Ethanol	9	12	14	13
Lantana camara	Leaf	Ethanol	NA	NA	11	9
Leonurus sibiricus	Leaf	Ethanol	10	8	14	NA
Maesa indica	Leaf	Dichloromethane	14	9	15	10
Ocimum sanctum	Leaf	Ethyl acetate	10	11	15	10
Polyalthia longifolia	Leaf	Ethyl acetate	11	20	23	15
Piper betle	Leaf	Dichloromethane	20	22	24	18
Syzygium cumini	Leaf	Ethyl acetate	9	10	14	12
Xanthium strumarium	Leaf	Benzene	9	14	16	12

*Diameter of inhibition zones tabulated was for 5mg/ml concentration of crude extracts.
****C.e.* = *Curvularia eragrostidis*, *L.t.* = *Lasiodiplodia theobromeae*, *C.c.* = *Colletotrichum camelliae*, *P.t.* = *Pestalotiopsis theae*

Table: 3.15. Bioautography of crude leaf-extracts of different plants showing antifungal activity against tested fungal pathogen of tea

Plants	Types of Extracts	Antifungal zones of inhibition (cm)				
		No. of zones	R_f	Zone of inhibition		
				C.e.	C.c.	L.t.
X. strumarium	Benzene	2	0.56	3.2	3.8	3.5
			0.25	2.5	3.2	1.5
C.excavata	Ethyl acetate	2	0.39	2.5	2.5	2.0
			0.30	1.5	1.5	0.6
P. longifolia	Ethyl acetate	1	0.73	2	2.5	1.5
P. betle	Dichloromethane	1	0.64	3.5	3.8	3.1
O. sanctum	Ethyl acetate	1	0.85	1.5	1.5	1.5

*****_C.e. = Curvularia eragrostidis_, _L.t. = Lasiodiplodia theobromeae_, _C.c. = Colletotrichum camelliae_

3.3.2. Bioactive compounds identified in *Clausena excavata* extracts

3.3.2.1. Excavarin-A (E-1):

A novel γ-lactone coumarin named as excavarin-A (**E-1**) was isolated from *C. excavata* leaf extracts as colorless needle shaped crystals. The compound produced blue-black spot at R_f 0.39 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The molecular formula was determined as $C_{19}H_{18}O_5$ by ESI-MS and molecular ion peak at m/z 327 $(M+H)^+$. IR spectrum showed the presence of a five–membered lactone ring (ν_{max}1742) and carbonyl group (1720cm^{-1}). The structure of the compound was confirmed by the ^1H and ^{13}C NMR data compared with published spectroscopic data (Thuy et al., 1999; Ito et al., 2000; Takemura et al., 2000). The ^1H NMR spectrum showed characteristic signals at δ_H 6.25, 7.64 (each 1H, d, *J*=9.6Hz), 7.38(1H, d, *J*=8.4Hz) and 6.85 (1H,

dd, *J*=8.6, 2.1Hz) for H-3, H-4, H-5 and H-6 respectively indicating the presence of coumarin nucleus. The side chain of this coumarin structure was elucidated by comparing its ^{1}H and ^{13}C NMR spectra with that of the known compound clauslactone-E (Ito et al., 2000). The ^{1}H NMR signal δ_H 4.62 (2H, d, *J*=6.3Hz, H-1'), 5.62-5.65 (1H, m, H-2'), 1.83 (3H, s, H-4'), 2.54 (1H, dd, *J*=14.1, 7.5Hz, H_a-5') and 2.38 (1H, dd, *J*=14.3, 5.4Hz, H_b-5') and ^{13}C NMR peak at δc 65.0, 122.7, 136.5, 17.3 and 45.7 indicated the presence of side chain of –O-CH_2-CH=C(CH_3)-CH_2-. Further, a carbonyl carbon signal of lactone ring at δ_C 170.0 and a methylene signal at δ_C 122.5 and δ_H 6.24, 5.65 (1H, t, *J*=3.0Hz) indicated that these moieties were attached to the side chain of lactone ring. The significant difference between **E-1** and clauslactone-E is absence of hydroxyl group at position C-8 (^{13}C-NMR signal at δ_C 101 instead of δ_C 133.3) (Ito et al., 2000). It also differed from excavatin-D, which did not contain methylene group attached to the lactone ring (Kongkathip et al., 2005). Based on all the above data, it was concluded that the structure of the isolated coumarin is as depicted in **E-1.** This was a novel compound and was named as excavarin-A. The nearest structure was reported in literature is clauslactone-E (Ito et al., 2000).

3.3.2.2. Excavatin-I (E-2):

Excavatin-I (**E-2**) isolated from leaf extracts was found as white crystal powder, m.p. 142-145^{o}C (uncorrected). The compound produced blue-black spot at R_f 0.30 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The UV absorption of purified compound was determined λ_{max} 209 and 328nm. The molecular formula was fixed at $C_{19}H_{20}O_6$ by ESI-MS and molecular ion peak at m/z 343 [M^+]. IR spectrum revealed the presence of hydroxyl group (3400 cm^{-1}), carboxyl group (1696 cm^{-1}) and five membered lactone carbonyl group (1734 cm^{1}). The final structure of the compound was elucidated by considering all the spectroscopy data; ^{1}H and ^{13}C NMR data showed close agreement with previously reported NMR data (Thuy et al., 1999). The ^{1}H NMR spectrum signal of **E-1** at δ_H 6.26 (d, *J*=9.2Hz, H-3), 7.65 (d, *J*=9.6Hz, H-4), 7.38 (d, *J*=8.8Hz, H-5), 6.85 (dd,

J= 8.8Hz, H-6) and 6.81 (s, H-8) are indicative of the presence of coumarin nucleus (Table 3.16). Proton signals at δ_H 4.62 (d, *J*=6.0 Hz, H-1'), 5.59 (t, *J*=6.0, H-2'), 2.53 (dd, *J*=8.0, 14.4 Hz, H-4'), 4.81 (m, H-5') and 1.85 (s, H-10') and ^{13}C NMR signal at δc 65, 122.4, 136.7, 44.8 and 17.1 showed the presence of –OCH_2-C=C(CH_3)-CH_2- side chain.

Table: 3.16. A comparative analysis of ^{1}H and ^{13}C NMR of excavarin-A (**E-1**) and excavatin-I (**E-2**) from *Clausena excavata* extract

Carbon	**Excavarin-A (E-1)**		**Excavatin-I (E-2)**	
	^{13}C	**^{1}H**	**^{13}C**	**^{1}H**
2	161.2	--	161.4	--
3	113.1	6.25 (d, *J*=9.3 Hz)	113.0	6.26 (d, *J*=9.2Hz)
4	143.4	7.64 (d, *J*=9.6 Hz)	143.5	7.65 (d, *J*=9.6Hz)
5	128.8	7.38 (d, *J*=8.4 Hz)	128.8	7.38 (d, *J*=8.8Hz)
6	122.3	6.85 (dd, *J*=8.6, 2.1 Hz)	113.1	6.85 (dd, *J*=8.8, 1.6Hz)
7	161.8	--	161.8	--
8	101.6	6.80 (d, *J*=2.1 Hz)	101.5	6.81 (d, *J*=1.9Hz)
9	155.8	--	155.7	--
10	112.6	--	112.5	--
1'	65.0	4.62 (d, *J*=6.3 Hz)	65.0	4.62 (d, *J*=6.0Hz)
2'	122.8	5.56-5.65 (m)	122.4	5.59 (t, *J*=6.0Hz)
3'	136.5	--	136.7	--
4'	17.3	1.83 (s)	17.2	1.83 (s)
5'	45.7	2.54 (dd, *J*=14.1, 7.5 Hz) 2.38 (dd, *J*=14.3, 5.4 Hz)	44.8	2.54 (dd, *J*=14.4, 8.0Hz) & 2.37 (dd, *J*=14.0, 5.2Hz)
6'	75.5	4.66-4.74 (m)	76.2	4.81 (m)
7'	33.1	3.06 (ddt, *J*=17.0, 7.8, 3.0 Hz) 2.64 (ddt, *J*=17.0, 5.6, 3.0 Hz)	42.7	2.46 (dd, *J*=13.6, 5.6Hz) 1.8 (d, *J*=7.2Hz)
8'	134.1	--	73.6	--
9'	170.0	--	177.7	--
10'	122.5	6.24 (t, *J*=3.0 Hz)	24.0	1.52 (s)

Excavatin-I (**E-2**) and Excavarin-A (**E-1**), the two antifungal compounds isolated from the leaves of *C. excavata* showed several similarities in their structures. For example, both compounds had the same coumarin nucleus and a side chain at similar position (C-7). However, their side chains showed some dissimilarity. Two prominent singlet signal at δ_H 1.83 and 1.52 in **E-2** (Fig. 3.10) indicated the presence of two methyl protons at C-4' and C-10' respectively. However, Excavarin-A showed a methylene signal at C-4' (δ_C 122.5) which was not observed in this compound. ^{13}C NMR signal at δc 177.7 (C-9'), 73.6 (C-8'), 42.7 (C-7') and 76.2 (C-6') observed in **E-2** were assigned to carboxylic group, α, ß and γ carbons of lactone ring respectively.

3.3.2.3. Dentatin (CR-1):

Dentatin (**CR-1**) was isolated from the root bark extracts of *C. excavata* as Colorless plates; m.p. 92-94^{o}C (uncorrected). The UV spectrum showed absorption at λ_{max} 205, 229, 272, 339 and 347 nm. The compound produced purple spot at R_f 0.49 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The molecular formula, $C_{20}H_{22}O_4$ was assigned by ESI-MS and molecular ion at m/z 327 $[M+H]^+$. IR bands showed peak at 1729 cm^{-1} which revealed the presence of carbonyl group (Songsiang et al., 2012); ^{1}H NMR signal resonating at δ_H 1.45 (6H, s, 2xCH_3, H-16, H-17), 1.66 (6H, s, 2xCH_3, H-14, H-15), 6.19 (1H, d, *J*=9.6, H-3), 7.87 (1H, d, *J*=9.6 Hz, H-4), 6.57 (1H, d, *J*=9.9Hz, H-6), 6.30 (1H, dd, *J*=17.4 & 10.8Hz, H-12), 5.71 (1H, d, *J*=9.9Hz, H-7), 4.94 (1H, d, *J*=17.4Hz, C-13a) and 4.88 (1H, d, *J*=10.5, H-13b) (Table 3.18) and the ^{13}C NMR signals at δc 160.8 (C-2), 156.2 (C-9a), 154.1 (C-5), 151.4 (C-10a), 150.0 (C-12), 139.0 (C-4), 130.7 (C-7), 119.3 (C-10), 116.5 (C-6), 111.8 (C-3 and C-5a), 108.3 (C-13), 107.7 (C-4a), 77.5 (C-8), 63.5 (OCH_3), 41.3 (C-11), 29.4 (C-14, C-15) and 27.7 (C-16, C-17) showed close agreement with previously reported NMR data (Songsiang et al., 2012). By considering all the spectroscopy data, the final structure of the compound was elucidated as dentatin (Fig. 3.10)

3.3.2.4. Nordentatine (CR-2):

Nordentatine (**CR-2**) was isolated from the root bark extracts as pale yellow amorphous solid; m.p. 179-180^{o}C (uncorrected). The compound produced blue spot at R_f 0.18 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The molecular formula was determined as $C_{19}H_{20}O_4$ by ESI-MS and molecular ion peak at m/z 313 $(M+H)^+$. The UV spectrum showed absorption at λ_{max} 228, 279 and 339 nm. IR (KBr) absorption showed at 3245, 2974, 1687, 1594, 1559 and 1464 cm^{-1} which was similar to that obtained by Songsiang et al. (2012). ^{1}H NMR ($CDCl_3$) showed characteristics signals at δ_H 8.10 (1H, d, *J* = 9.6 Hz, H-4), 6.67 (1H, d, *J* = 9.9 Hz, H-6), 6.38 (1H, s, OH), 6.30 (1H, dd, *J* = 17.2, 10.5 Hz, H-20), 6.1 (1H, d, *J* = 9.6 Hz, H-3), 5.62 (1H, d, *J* = 9.9 Hz, H-7), 4.94 (1H, d, *J* = 17.7 Hz, H-13a), 4.84 (1H, d, *J* = 10.5 Hz, H-13b), 1.63 (6H, s, 2xCH_3, H-14, H-15) and 1.43 (6H, s, 2xCH_3, H-16, H-17). The ^{13}C NMR ($CDCl_3$) resonating at 162.4 (C-2), 156.4 (C-9a), 154.1 (C-10a), 150.3 (C-12), 148.1 (C-5), 140.5 (C-4), 128.9 (C-7), 116.3 (C-10), 115.4 (C-6), 109.3 (C-3), 107.9 (C-13), 107.1 (C-5a), 104.6 (C-4a), 77.6 (C-8), 41.0 (C-10), 29.5 (C-14 and C-15) and 27.3 (C-16, C-17) was found to match with that of Nordentatin (Songsiang et al., 2012). The detailed spectroscopic data (Table 3.18) was analysed and it was concluded that the isolated compound was Nordentatin (Fig 3.10).

3.3.2.5. Clausenidin (CR-3):

Clausenidin (**CR-3**) (Fig. 3.10) was isolated from the root bark extracts of *C. excavata* as pale yellow powder; m.p. 148-150^{o}C (uncorrected). The compound produced cyan blue spot at R_f 0.63 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The molecular formula, $C_{19}H_{20}O_5$ was assigned by ESI-MS and molecular ion at m/z 329 $[M]^+$. The UV spectrum showed absorption at λ_{max} 205, 219, 285 and 329 nm. IR bands showed at 3437 (hydroxyl group), 2980, 1734 (carbonyl group), 1604 and 1374 cm^{-1} (Wu and Furukawa, 1982). ^{1}H NMR signal resonating at δ_H 1.49 (6H, s, 2xCH_3, H-16, H17), 1.64 (6H, s, 2xCH3, H-14, H-15), 6.16 (1H, d, *J*=9.6Hz, H-3), 8.04 (1H, d, *J*=9.9Hz, H-4), 12.98

(s, 5-OH), 2.75 (2H, s, H-7), 6.23 (1H, dd, *J*=17.4 & 9.6Hz, H-12), 4.92 (1H, d, *J*=15Hz, H-13a) and 4.87 (1H, d, *J*=8.1Hz, H-13b) were observed. The ^{13}C NMR (Table 3.18) peak was observed at δc 198.4 (C-6), 160.8 (C-2), 160.1 (C-9a), 159.2 (C-5), 149.7 (C-10a), 138.7 (C-12), 138.7 (C-4), 114.6 (C-10), 110.9 (C-3), 108.5 (C-5a), 108.5 (C-13), 104.2 (C-4a), 80.2 (C-8), 47.8 (C-7), 41.1 (C-11), 29.6 (C-14, C-15) and 26.6 (C-16, C-17). The broad IR band at 3437 cm^{-1} and low field ^1H NMR peak at δ 12.98 ppm indicated a strongly hydrogen bonded phenolic proton (Huang et al., 1997).

3.3.2.6. Clausarin (CR-5):

Clausarin (**CR-5**) was isolated from the root bark extracts of *C. excavata* as colourless crystals. The compound produced green spot at R_f 0.55 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17).The UV spectrum taken in methanol showed absorption with λ_{max} at 210, 229, 281 and 334 nm. IR absorption showed at ν_{max} 3137 and 1668 cm^{-1} that were assigned as hydroxyl and carbonyl groups respectively. The molecular formula, $C_{24}H_{28}O_4$ was assigned by ESI-MS as molecular ion peak at m/z 381 $[M+H]^+$. ESI-MS spectral data of clausarin further showed that the cleavage of side chain, 3-dimethyl allyl group at C-3 position, resulted in the formation of nordentatin with a peak at m/z 311. The additional fragments at m/z 244 showed the removal of both 3-dimethyl allyl groups from C-3 and C-10 position and at m/z365 showed removal of hydroxyl group at C-5 position.

The ^1H-NMR spectrum in $CDCl_3$ (300Hz) showed several characteristic peaks at δ_H 1.42 (s, 2XCH_3 , H-4', H-5') 1.47 (s, 2XCH_3, H-14, H-15), 1.63 (s, 2XCH_3, H-16, H-17), 4.84 (1H, d, H-3'a, *J*=10 Hz), 4.92(1H, d, H-3'b, *J*=17.6 Hz), 5.07 (1H, d, H-13a, *J*=2.4 Hz), 5.09 (1H, d, H-13'b, *J*=10 Hz), 5.66 (1H, d, H-7, *J*=9.6Hz), 5.99 (s, OH, H-5), 6.17 (1H, dd, H-2', *J*=17.6 and 10.4 Hz), 6.28 (1H, dd, H-12, *J*=17.6 and 10.4), 6.54 (1H, d, H-6, *J*=9.6Hz) and 7.88 (1H, s, H-4). ^{13}C NMR($CDCl_3$) showed peaks at δ_C 26.08 (2xCH_3, C-4', H-5'), 27.2 (2xCH_3, C-16, C-17), 29.4 (2xCH_3, C-14, C-15), 40.0 (C-1') 40.9 (C-11), 77.3 (C-8), 104.0 (C-4a),

105.5 (C-5a), 107.9 (C-13), 111.9(C-3'), 115.3 (C-6), 115.4 (C-10), 133.3 (C-4), 128.9 (C-7), 129.7 (C-3), 145.6 (C-2'), 150.1 (C-12), 153.3 (C-10a), 154.8 (C-9a) and 160 (C-2). The NMR spectral data (Table 3.18) of clausarin (Fig. 3.10) matched with previously published data (Huang et al., 1997; Peng et al., 2013). The spectral data was very close to nordentatin, but differed in the ^{1}H NMR spectral data at δ_H 7.88 (s) (C-3) instead of 8.10 (d) (in nordentatin) suggesting the substitution by 3-dimethyl allyl group at C-3 position.

3.3.2.7. Osthol (CR-4)

Osthol (**CR-4**) was isolated from the root bark extracts of *C. excavata* as brown powder. The UV spectrum of the bio-active compound was taken in methanol solution. The UV absorption was observed with λ_{max} at 206, 225, 269 and 329 nm. The compound produced green spot at R_f 0.38 when run in TLC and sprayed by anisaldehyde-H_2SO_4 (Table 3.17). The infrared absorption spectrum (in KBr) was observed mainly at ν_{max} 3352, 1728 (C=O) and 1615 cm^{-1}. The ^{1}H-NMR spectrum in $CDCl_3$ (300Hz) showed several characteristic peaks at δ_H: 1.66 (3H, s, H-13), 1.83 (3H, s, H-12), 3.53 (2H, d, *J*=7.2, H-9), 3.92 (3H, s, OCH_3), 5.22 (1H, t, *J*=7.2, H-10), 6.19-6.26 (1H, m), 6.83 (1H, d, *J*=8.4, H-6), 7.28 (1H, d, *J*=10.5, H-5) and 7.84 (1H, d, *J*=9.6Hz, H-4) that led to assume that the structure of the compound may be **CR-4**. A sharp singlet appearing at δ_H 3.92 ppm indicated the presence of one methoxy group attached with aromatic carbon (C-7). Additional two sharp singlets appearing at δ_H 1.83 and 1.66 ppm revealed the presence of two methyl groups, which could be assigned to C-12 and C-13.

The ^{13}C-NMR spectrum also supported the structure of the compound **CR-4** (Thongthoom et al., 2010). Thus, three aliphatic carbons were displayed in the range of δ_C 18.1-29.8 ppm and ten olefinic carbons appeared in the range of δ_C 107.6–157.0 ppm. A sharp peak observed at δ_C 56.2 revealed the presence of methoxy group at C-7. In addition, two peaks at δ_C 121 and 132 ppm indicating aliphatic double bond between C-10 and C-11 was found. Similar observations were reported by Thongthoom et al. (2010) in their studies on structure

identification of antifungal compound. Unluckily, because of low concentration of compound, the carbonyl carbon was difficult to recognize from noises. However, combination of all spectral data followed by analyses, as depicted above, led us to tentatively propose that the structure of the antifungal component may have the chemical structure of osthol (Fig. 3.10).

Table: 3.17. Phytochemical properties of compounds isolated from *Clausena excavata* extracts detected by TLC following exposure to UV light and reagents

Phytochemical components	**Type**	**R_f**	**UV_{254nm}**	**UV_{365nm}**	**Anisaldehyde -H_2SO_4 (Spray reagent)**
Excavarin-A	Furano-Coumarins[a]	0.39	Greenish	Blue	Blackish blue
Excavatin-I		0.30	Greenish	Blue	Blackish blue
Clausenidin	Coumarins[b]	0.63	Deep green	-[c]	Cyan Blue
Dentatin		0.49	Deep Blue	Blue	purple
Nordentatin		0.18	Gray	Blue	Blue
Osthol		0.38	Faint Blue	Blue	Green
Clausarin		0.55	Green	-[c]	Green

[a]Hexane : ethyl acetate=60:40 or [b]hexane : ethyl acetate=70:30; [c]no characteristic colour observed

Table: 3.18. ^{1}H & ^{13}C NMR data of clausinidin, dentatin, nordentatin and clausarin from root bark of *Clausena excavata.*

Carbon	Clausenidin		Dentatin		Nordentatin		Clausarin	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	160.8	--	160.8	--	162.4	--	160.0	--
3	110.9	6.16(d, *J*=9.6Hz)	111.8	6.19 (d, *J*=9.6Hz)	109.3	6.09 (d, *J*=9.6Hz)	129.7	--
4	138.7	8.04 (d, *J*=9.9Hz)	139.0	7.87 (d, *J*=9.6 Hz,	140.5	8.10 (d, *J*=9.6Hz)	133.2	7.88 (s)
4a	104.2	--	107.7	--	104.6	--	104.0	--
5	159.2	12.98 (s, OH)	154.1	3.83 (s) -OCH_3	148.1	7.3 (s, OH)	146.8	5.99 (s, OH)
5a	108.5	--	111.8	--	107.1	--	105.9	--
6	198.4	--	116.5	6.57 (d, *J*=9.9Hz)	115.4	6.67 (d, *J*=9.9Hz)	115.3	6.54 (d, *J*=9.6Hz)
7	47.8	2.75 (s)	130.5	5.71 (d, *J*=9.9Hz)	128.9	5.62 (d, *J*=9.9Hz)	128.9	5.66 (d, *J*=9.6Hz)
8	80.2	--	77.5	--	77.1	--	77.3	--
9a	160.1	--	156.2	--	156.4	--	154.8	--
10	114.6	--	119.3	--	116.3	--	115.4	--
10a	149.7	--	151.4	--	154.1	--	153.2	--
11	41.1	--	41.3	--	41.0	--	40.2	--
12	138.7	6.23 (dd, *J*=17.4 & 9.6Hz)	150.0	6.30 (dd, *J*=17.4 & 10.8Hz	150.3	6.30 (dd, *J*=17.2 & 10.5Hz)	150.1	6.28 (dd, *J*=17.6 & 10.4)
13	108.5	4.92 (d, *J*=15Hz) 4.87 (d,*J*=8.1Hz)	108.3	4.94 (d, *J*=17.4Hz) 4.88 (d, *J*=10.5Hz)	107.9	4.91 (d, *J*=17.7Hz) 4.84 (d, *J*=10.5Hz)	107.9	5.07 (d, *J*=2.4Hz), 5.09 (d, *J*=10 Hz)
14	29.6	1.64 (s, CH_3)	29.6	1.66 (s)	29.6	1.63 (s)	29.4	1.47 (s)
15	29.6	1.64 (s, CH_3)	29.6	1.66 (s)	29.6	1.63 (s)	29.4	1.47 (s)
16	26.6	1.49 (s, CH_3)	27.7	1.45 (s)	27.4	1.43 (s)	27.1	1.63 (s)
17	26.6	1.49 (s, CH_3)	27.7	1.45 (s)	27.7	1.43 (s)	27.1	1.63 (s)
1'	--	--	--	--	--	--	40.9	--
2'	--	--	--	--	--	--	145.6	6.17 (dd, *J*=17.6 & 10.4 Hz)
3'	--	--	--	--	--	--	111.9	4.84 (d, *J*=10 Hz), 4.92(d, *J*=17.6 Hz)
4'	--	--	--	--	--	--	26.1	1.42 (s)
5'	--	--	--	--	--	--	26.1	1.42 (s)

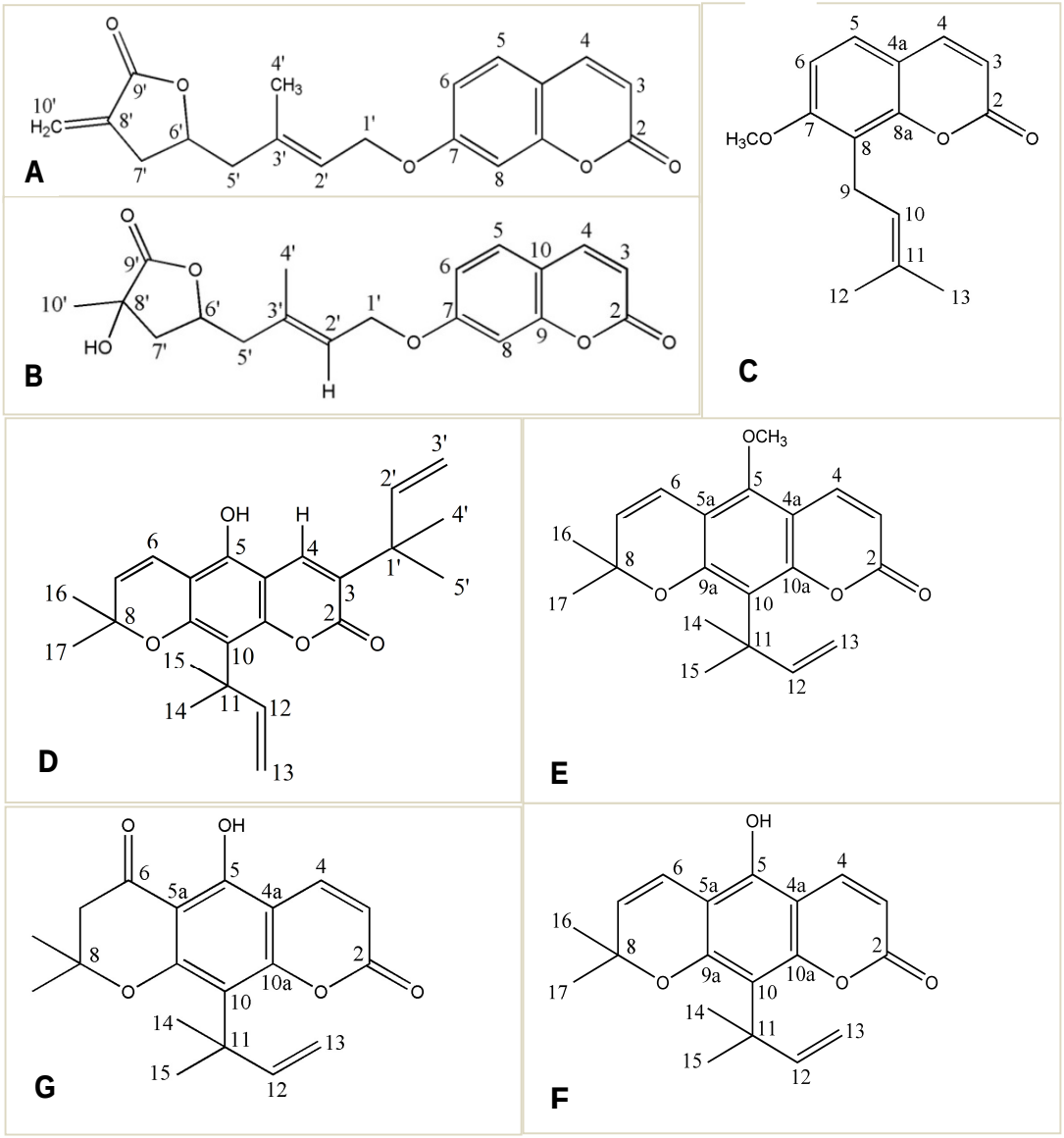

Fig. 3.10. Isolated phytochemical compounds from *Clausena excavata*

A: Excavarin-A from *C. excavata* leaf

B: Excavatin-I from *C. excavata* leaf

C: Osthol from *C. excavata* root bark

D: Clausarin from *C. excavata* root bark

E: Dentatin from *C. excavata* root bark

F: Nordentatin from *C. excavata* root bark

G: Clausenidin from *C. excavata* root bark

3.3.3. Antifungal activity

Bioactivity of the extract was monitored by bioautography; the presence of antifungal compound was evident by the appearance of a clear zone of inhibition. The purified compounds were evaluated for antifungal activity against fungal strains by the 96-well microtitre plate assay and activity was compared to the standard fungicides, bavistin and nystatin. Test compounds were serially diluted and subsequently mixed with potato dextrose broth and inoculum in microtitre plates to obtain eight dilutions (2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 mg/ml). The result of MIC evaluation is given in tables 3.19.

Antifungal activity of excavarin-A was found to be stronger than that of the standard antibiotic nystatin against the clinically important pathogens, *A. fumigatus, C. tropicalis* and *M. circinelloides*. The plant pathogenic fungi, *C. gloeosporioides, C. eragrostidis, F. oxysporum, L. theobromae, R. solani, R. stolonifer* and *S. sclerotiorum* were more sensitive towards the isolated compound than the standard fungicide bavistin. *R. solani* and *F. oxysporum* were found to be the most sensitive strains with an MIC value less than 0.019 mg/ml. Fungicidal action against *C. curvatus* and *T. cutaneum* was observed to be similar to the standard antibiotic. *A. niger* and *A. fumigatus* were found to be most resistant (MIC 0.625 mg/ml). The antagonistic potential of excavarin-A was found to be considerably strong which may be due to the α-methylene-γ-lactone moiety present in the side chain. Such systems are implicated in bioactivity in the sesquiterpene lactones (Wedge et al., 2000). However, α, ß-unsaturated carbonyl moiety makes the sesquiterpene lactones reactive towards thiol groups of proteins (Schmidt, 1999). Therefore, further studies on probable toxicity of excavarin-A are necessary before developing it as a therapeutic agent.

Moderate to weak antifungal activity was noted in excavatin-I which is a new observation. The compound showed weaker antifungal activity than standard fungicide bavistin except against *C. camelliae* (Table 3.20). Maximum activity was observed against *C. camelliae* (0.39 mg/ml) and least activity was observed against *F. oxysporum* (2.5 mg/ml). The compound was also weak in

activity when compared to excavarin-A. This may be due to the absence of α-methylene-γ-lactone moiety in the side chain. Due to its weak bioactivity, the compound was only tested for activity against tea pathogens and a few other plant pathogens.

Literature review has revealed that *C. excavata* have diverse group of coumarins with broad range of biological activities. However, till date, very few studies have been done concerning the antifungal activity of phytochemicals of *C. excavata*. Three compounds were reported to be isolated from rhizome of *C. excavata* namely 3-formylcarbazole, mukonal and 3-methoxycarbonylcarbazole which showed antifungal activities with IC_{50} values of 13.6, 29.3, 9.5 and 2.8 µg/ml, respectively (Sunthitikawinsakul et al., 2003). Anisucoumarin A and B isolated from leaves and twigs of *C. anisum-olens* which did not contain the α-methylene-γ-lactone moiety, failed to show antifungal activity against *Candida albicans, C. tropicalis* and *C. krusei* (Wang et al., 2008).

Among the compounds isolated from the roots bark of *C. excavata*, only osthol was found to be antifungal. The compound was found to be moderately antifungal against most of the tested plant pathogens (Table 3.21). However, exceptionally strong inhibition was observed against *Sclerotium rolfsii* which produced an MIC of 0.019 mg/ml. Among the tea pathogens, *L. theobromae* was found to be most susceptible (MIC: 0.078 mg/ml). In several instances, osthol performed better than the tested fungicide. Osthol is a natural coumarin derivative isolated from different sources such as *Clausena anisata* root (Okorie, 1975), root bark of *C. excavata* (Huang et al., 1997), *C. harmandiana* (Thongthoom et al., 2010), *C.guillauminii* (Nakamura et al., 2009), *Cnidium monnieri* (Liao et al., 2010), *Angelica archangelica*, *Phebalium* sp. (Rashid et al., 1992; Wei et al., 2004), dried fruits of *Cnidii monnieri* fructus (Shi et al., 2007), *Prangos asperula* (Sajjadi et al., 2009) and root of *Ferulago campestris* (Basile et al., 2009). Osthol has been reported to exhibit wide spectrum of antifungal activity, with the EC_{50} values ranging from 21.15 µg ml^{-1} to 61.62 µg ml^{-1} against

Botrytis cinerea, Rhizoctonia solani, *Colletotrichum musae, Fusarium graminearum, Phytophora capsici, Sclerotinia sclerotiorum* and other phytopathogenic fungi (Shi et al., 2004). The compound could inhibit the hyphal growth of *F. graminearum* by decreasing hyphal absorption to reducing sugar (Shi et al., 2008). Antifungal and prophylactic effect against pumpkin powdery mildew was observed on osthol application at a concentration of 100 µg ml^{-1} and it also induced a resistance response against powdery mildew in pumpkin leaves (Shi et al., 2007). Antifungal activity against *Candida albicans* with IC_{50} value 19.40 was reported by Thongthoom et al. (2010). Besides antifungal activity, osthol was found to possess several biological activities such as antitumor (Chou et al., 2007), antifeedant (Pitan et al., 2009), anti-inflammatory (Nakamura et al., 2009; Liao et al., 2010; Shen et al., 2012) and neuroprotective (He et al., 2012).

Table: 3.19. Antifungal activity of excavarin-A (**E-1**) purified from leaf extract of *Clausena excavata*

Tested fungal pathogens	Strain No.	Minimum inhibitory Concentration in mg/ml		
		Excavarin-A	Bavistin	Nystatin
Plant pathogens				
**Colletotrichum gloeosporioides*	ITCC 5446.02	0.039	0.078	-[a]
**Curvularia eragrostidis*	ITCC 4150.2K	0.312	1.25	-
**Lasiodiplodia theobromae*	ITCC 4151.2K	0.039	0.078	-
**Rhizoctonia solani*	ITCC 5995.05	<0.019	0.039	-
Fusarium oxysporum	ITCC 6246	0.019	2.5	-
Rhizopus stolonifer	ITCC 6283	0.019	0.039	-
Sclerotinia sclerotiorum	ITCC 6094	<0.019	0.078	-
Human pathogens				
Aspergillus niger	MTCC 2425	0.625	-[a]	0.156
Aspergillus fumigatus	MTCC 6594	0.625	-	1.25
Candida albicans	MTCC 183	0.078	-	0.039
Candida tropicalis	MTCC 184	0.039	-	0.625
Cryptococcus curvatus	MTCC 2698	0.078	-	0.078
Filobasidiella neoformans	MTCC 1431	0.078	-	0.019
Mucor circinelloides	MTCC 3944	0.078	-	0.156
Trichosporon cutaneum	MTCC 1963	0.078	-	0.078

[a] '-' = Not tested, * =Tea pathogens

Table: 3.20. Antifungal activity of excavatin-I (**E-2**) purified from leaf extract of *Clausena excavata*

Tested fungal pathogens	Strain No.	Minimum inhibitory Concentration (MIC) mg/ml	
		Excavatin-I	Bavistin
**Colletotrichum camelliae*	CC01	0.039	0.078
**Curvularia eragrostidis*	ITCC 4150.2K	1.25	1.25
Fusarium oxysporum	ITCC 6246	2.5	2.5
**Lasiodiplodia theobromae*	ITCC 4151.2K	1.25	0.078
Rhizopus stolonifer	ITCC 6283	0.312	0.039
Sclerotinia sclerotiorum	ITCC 6094	0.156	0.078

* =Tea pathogens

Table: 3.21. Antifungal activity of osthol isolated from root bark extract of C*lausena excavata* against tested fungal pathogens

Tested pathogens	Minimum inhibitory concentration in mg/ml		
	Osthol	Bavistin	Nystatin
Plant pathogens	0.312	0.078	-[a]
**Colletotrichum camelliae*			
**Curvularia eragrostidis*	0.312	1.25	-
**Lasiodiplodia theobromae*	0.078	0.078	-
**Pestalotiopsis theae*	0.156	0.312	-
Fusarium oxysporum	0.312	2.5	-
Fusarium equiseti	0.078	0.156	-
Rhizoctonia solani	0.625	0.039	-
Rhizopus stolonifer	0.156	0.039	-
Sclerotinia sclerotiorum	0.156	0.078	-
Sclerotium rolfsii	0.019	0.078	-
Macrophomina phaeseolina	0.312	0.312	-
Human pathogens			
Mucor circinelloides	0.312	-[a]	0.156
Candida albicans	0.625	-	0.039
Candida tropicalis	1.25	-	0.625
Filobasidiella neoformans	0.156	-	0.019

[a]'-'= Not tested, * =Tea pathogens

3.3.4. Antioxidant and DNA protective activity

Among the isolated compounds of *C. excavata*, only three were found to be antifungal. In order to study the bioactive potential of other compounds, they were tested for their reducing potential and free radical scavenging activity due to the presence of the phenolic –OH groups in two compounds. Nordentatin and clausarin showed reducing potential while the other compounds, clausenidin, dentatin and osthol did not reduce even at 1250µg ml^{-1} concentration (Fig. 3.11).

The TLC-based qualitative DPPH spray revealed the presence of significant antioxidant activity in clausarin and nordentatin only indicated by the presence of a yellowish spot on the reddish purple back ground of the TLC plate (Fig. 3.12).

These compounds also showed a good free radical scavenging activity at all tested concentrations. The scavenging activity increased with an increase in concentration of nordentatin and clausarin as well as standard compound ascorbic acid (Fig. 3.11). However, both clausarin and nordentatin showed higher activity than ascorbic acid with clausarin showing higher reducing potential than nordentatin. These compounds were found to be a better electron donor than ascorbic acid and could react with free radicals to convert them to more stable products and terminate radical chain reaction (Wu et al., 2011). The antioxidant activity of nordentatin isolated from root bark of *Clausena harmandiana* has been previously reported by Songsiang et al. (2012). However, there is no earlier report of clausarin showing antioxidant activity. Clausarin showed more reducing power than nordentatin which might be due to the presence of extra dimethyl allyl group at C-3 position which has positive inductive effect.

Reducing power of plant compounds which is determined by their capacity to reduce Fe^{+3} to Fe^{+2} (Meir et al. 1995) *in vitro* are generally due to the presence of di- and mono-hydroxyl substitution in the aromatic ring which possesses potential hydrogen donating ability (Mathew and Abraham, 2006). The hydroxyl group acts as a reductant, which exerts antioxidant activity by breaking the radical chain by donating a hydrogen atom (Subhan et al., 2008).

The reducing power of clausarin and nordentatin (Fig. 3.13) might be due to the presence of the hydroxyl group in the aromatic ring of coumarin nucleus at C-5 position, which can donate the hydrogen. The reduction capability of DPPH radicals was determined by a decrease in absorbance at 517 nm induced by antioxidants (Emen et al., 2009; Chang et al., 2007; Huang et al., 2005). DPPH in free radicals is purple in color and after accepting hydrogen radical it forms stable diamagnetic molecule which turns yellow (Patel and Patel, 2011). Clausarin and nordentatin could act as reducing agent by reducing the DPPH free radicals by donating hydrogen from hydroxyl group of C-5. Clausenidin also had a hydroxyl group at C-5 position but did not exhibit reducing power most probably due the formation of strong intramolecular hydrogen bond between hydroxyl group and neighboring carboxyl group at C-6 position.

In the current study, the DNA damage protective activities of tested compounds were investigated with pUC18 plasmid DNA. The protective activity was evaluated on agarose gel and the patterns of plasmid DNA were seen after irradiation of UV light (Fig. 3.14). Two intense band of plasmid DNA were observed on agarose gel, the faster moving band corresponded to the native form of supercoiled circular DNA (scDNA) in lane 1 and the slower moving band was the open circular form (ocDNA) in lane 2. Super-coiled plasmid DNA converts into linear form by the cleavage of the scDNA in presence of H_2O_2 (lane 2). It is indicated that, the hydroxyl free radicals generated from UV irradiated photolysis of H_2O_2 caused DNA strand scission (Boubaker et al., 2011). The addition of nordentatin and clausarin (Fig. 3.14 in lanes 3, 4 & 7) to the reaction mixture suppressed the formation of linear DNA and induced the recovery of scDNA. Compounds, clausenidin, dentatin and osthol did not show DNA damage protective potential. When compared with the standard compounds, quercetin, nordentatin and Clausarin showed better protective potential. This study has shown for the first time that the natural antioxidant compound nordentatin and clausarin have capacity to protect DNA from oxidative damage by free radical scavenging.

3.3.5. Antifungal compounds identified in *Xanthium strumarium* leaf extracts

3.3.5.1. 8-*epi*-Xanthatin (X1):

Bioactive **X-1** was purified from *X. strumarium* leaf extract after repeated silica gel column chromatography. The compound was obtained by re-crystallization in ethyl acetate and hexane to obtained white needle like crystal. The UV spectrum of crystallized compound was taken in methanol solution. A maximum absorbance with λ_{max} at 271 nm was observed, indicating the presence of lactone structure. The compound produced an R_f at 0.56 when run in TLC and showed reddish brown and brown colour spots when sprayed by anisaldehyde-H_2SO_4 and Vanilin-H_2SO_4 respectively indicating sesquiterpene (Table 3.22). The IR spectrum were observed mainly at 1751, 1750, 1595 cm^{-1}, which revealed the presence of carbonyl groups (corresponding to keto and lactone carbonyl) and C=C double bond respectively. The molecular formula of $C_{15}H_{18}O_3$ was deduced from the ESI-MS data which showed molecular ion peak at m/z 269 $[M+Na]^+$ and was further confirmed by ^{13}C and ^{1}H NMR (Table 3.23), HMQC, DEPT 45 and DEPT 135 data analysis.

The ^{1}H NMR spectrum showed several characteristic signals; δ_H at 6.99 (d, *J*=16.2 Hz) and 6.14 (d, *J*=16.2 Hz) indicated two olefinic protons coupled to each other as *vicinal* coupling attached with H-2 and H-3 having *trans* configuration (as noticed from high value of coupling constant, *J*). On the other hand, two vinyl protons at C-13 appeared as doublets (geminal coupling) at δ_H 5.57 (d, *J*=3.0) and 6.30 (d, *J*=3.3) ppm. These two vinyl protons attached at H-13 should have different chemical shifts because of the rigidity around the double bond and different electronic environments. A sharp peak appearing at δ_H 2.29 (s) ppm indicated the presence of one methyl group (H-15) attached with carbonyl group. Further occurrence of the methyl group was exhibited at δ_H 1.18 (d, *J*=6.6) ppm, which is assigned to H-14 methyl group.

The ^{13}C-NMR and DEPT spectrum analysis exhibited 15 carbon signals, including four quaternary carbons at 198.4, 169.7, 142.8 and 138.0, six methine

signals at 146.4, 135.6, 125.7, 78.1, 41.0 and 31.6, and three methylene signals at 122.4, 36.6 and 26.8 ppm. Two characteristic signals of methyl group were also found at δc 27.5 and 21.3 ppm which were assigned to the C-15 and C-14 respectively. On other hand, two unsaturated keto group signals were observed at δc 198.4 and 169.6 ppm, which were assigned respectively to C-4 and C-12 of lactone group. A C-13 olefinic carbon appeared at δc 122.6 ppm, as was also observed by Kim et al. (2002) in their studies on structural identification of antifungal compound extracted from *X. strumarium*. Chemical identity of **X-1** was established through analyses of its spectral data and the final structure of the bio-active compound revealed that the compound was 8-*epi*-xanthatin (Fig. 3.15). Previous isolation of xanthatin has been reported and our results corroborated with the investigation made by Kim et al. (2002) and Kanauchi et al. (1999).

3.3.5.2. 8-*epi*-xanthatin-1ß,5ß-epoxide (X-2):

Bioactivity guided fraction of dichloromethane leaf extract of *X. strumarium* led to the isolation of the compound 8-*epi*-**x**anthatin-1ß,5ß-epoxide after repeated silica gel column chromatography. The compound produced strong UV absorption at λ_{max} 229 nm. The compound was spotted at R_f 0.25 when run on TLC and showed characteristic colour indicating the presence of sesquiterpene (Table 3.22). Molecular formula $C_{15}H_{18}O_4$ and molecular ion peak at m/z 262 $[M-H]^+$ was determined by ESI-MS spectroscopy. The IR band at ν_{max} 1762 and 1672 cm^{-1} indicated the presence of α-methylene-γ-lactone ring and carbonyl group respectively. The structure was confirmed by the ^1H and ^{13}C NMR data when compared with previously published spectroscopic data (Cumunda et al., 1991). The ^1H NMR spectrum showed signal at 6.75(1H, d, *J*=15.9Hz) and 6.27 (1H, d, *J*=15.6Hz) which are characteristic for H-2 and H-3. This large coupling constant between the H-2 and H-3 represents that both proton must exist in trans conformation. In addition to a methyl singlet at δ_H 2.27 (3H, s) for H-15 of an acetyl group, other methyl doublets at δ_H 1.12 (3H, d, 6.9Hz) for H-14 were

found. The structure of the compound (**X-2**) was determined by comparing with the ^{1}H and ^{13}C NMR spectral data of the known compound 1ß, 5ß-epoxy-1,5,11α,13-tetrahydro-8-*epi*-xanthatin (Cumunda et al., 1991). The ^{13}C NMR peaks at δc 62.96 (C-1), 146.8 (C-2), 129.6 (C-3), 197.8 (C-4) and 28.39 (C-15) indicated the presence of –C-CH=CH-C-CH_3 side chain as was observed in 8-*epi*-xanthatin. The ^{13}C NMR peaks at δc 62.96 (C-1) and 66.0 (C-5) did not match with 8-*epi*-xanthatin, but was found to be similar to that of 1ß,5ß-epoxy-1,5,11α,13-tetrahydro-8-*epi*-xanthatin reported by Cumanda and Marinoni (1991) indicating the presence of epoxide ring. Characteristic peak of carbonyl carbon signal of α-methylene-γ-lactone ring at δc 169.2 (C-12) and a methylene signal at δc 123.5 (C-13), distinguished **X-2** (Fig. 3.15) from the compound 1ß,5ß-epoxy-1,5,11α,13-tetrahydro-8-*epi*-xanthatin.

The spectral data of 8-*epi*-xanthatin-1ß, 5ß-epoxide is close to 8-*epi*-xanthatin. However, a few signals were characteristically different. In particular the presence of a -1,5-epoxide, instead of the double bond present in 8-*epi*-xanthain, was obviously indicated by the upfield shift of the H-5 signal, which moved from δ_H 6.20 ppm for 8-*epi*-xanthatin to 3.07 ppm for 8-*epi*-xanthatin-1ß, 5ß-epoxide (Table 3.23). Systematically, the ^{13}C NMR spectra of 8-*epi*-xanthatin-1ß, 5ß-epoxide contained two ether linked carbons at C-1 (δ_C 66.0 ppm) and C-5 (δ_C 62.9 ppm) instead of the signals of the tri-substituted double bond (δc 138.0 and 135.6 for C-1 and C-5 respectively) of 8-*epi*-xanthatin (Table 3.23).

Table: 3.22. Phytochemical properties of compounds isolated from *Xanthium strumarium* leaf extract detected by TLC following exposure to UV light and reagent spray

Phytochemical	**Class of the phytochemical**	**R_f**	**UV_{254nm}**	**Spray reagents**	
				Anisaldehyde-H_2SO_4	**Vanilin-H_2SO_4**
8-*epi*-xanthatin	Sesquiterpene	0.56	Green	Reddish brown	Brown
8-*epi*-xanthatin-1ß, 5ß-epoxide		0.25	Green	Reddish brown	Brown

Table: 3.23. ^{1}H & ^{13}C NMR data analysis of phytochemicals 8-*epi*-xanthatin and 8-*epi*-xanthatin-1ß, 5ß-epoxide isolated from *X. strumarium*

Carbon	8-epi-Xanthatin (**X-1**)		8-epi-xanthatin-1ß,5ß-epoxide (**X-2**)	
	^{13}C	^{1}H	^{13}C	^{1}H
1	138.0	--	62.90	--
2	146.4	6.99 (H, d, *J*=16.2Hz)	146.8	6.75 (d, *J*=15.9 Hz)
3	125.7	6.14 (H, d, *J*=16.2Hz)	129.6	6.27 (d, *J*=15.6 Hz)
4	198.4	--	197.8	--
5	135.6	6.20 (H, dd, *J*=6.3 & 9.0Hz)	66.0	3.07 (dd, *J*=6 & 9 Hz)
6	26.9	2.18 (H_{6a}, ddd, *J*=2.1, 6.0 & 15.0Hz) 2.53 ($H_{6b,}$dt, *J*=2.4Hz)	32.19	1.21-1.88 (m)
7	41.1	3.37-3.46 (H, m)	39.4	2.62 (m)
8	78.2	4.66 (H, dt, *J*=6.3, 9.0Hz)	79.4	4.51 (dt)
9	36.2	1.84-1.97 (2H, m)	31.7	1.99-2.05 (m) & 1.7 (m)
10	31.6	2.79-2.87 (H, m)	31.4	2.39-2.79 (m)
11	142.8	--	139.1	--
12	169.7	--	169.2	--
13	122.4	6.30 ($H_{13a,}$ d, *J*=3.3Hz) 5.57 (H_{13b}, d, *J*=3.0Hz)	123.5	6.27 (d), 5.66 (d)
14	21.4	1.18 (3H, d, *J*=6.6Hz)	18.8	1.12 (d)
15	27.6	2.29 (3H, s)	28.39	2.26 (s)

3.3.6. Antifungal activity

Bioactivity of the extract was monitored by bioautography; the presence of antifungal compound was evident by the appearance of a clear zone of inhibition (Fig. 3.16). The purified compounds were evaluated for antifungal activity against fungal strains by the 96-well microtitre plate assay and activity was compared to the standard fungicides, bavistin and nystatin. Antifungal activity of both 8-*epi*-xanthatin and 8-*epi*-xanthatin-1ß, 5ß-epoxide was found to be comparable to that of the standard antibiotics (Table 3.24). All the tested tea pathogens were more sensitive towards the isolated compounds than the standard fungicide. 8-*epi*-xanthatin contained the α-methylene-γ-lactone moiety which is often implicated in bioactivity in the sesquiterpene lactones (Wedge et al., 2000). In general, 8-*epi*-xanthatin showed stronger antifungal activity than 8-*epi*-xanthatin-1ß, 5ß-epoxide.

Little et al. (1950) was the first to report the isolation of a compound with strong antimicrobial properties from *Xanthium pennsylvanicum* which was given the name xanthatin. Xanthatin has been identified from *X. strumarium* leaves as potent antimicrobial compound against several bacteria and yeast, and also useful for preventing contamination in koji and during production of alcoholic beverage (Kanauchi et al., 1999). Though the medicinal properties of *X. strumarium* are well known, it has not been exploited for use as an agricultural product.

Kim et al. (2002) has shown that deacetyl xanthumin, which structurally resenmbled 8-*epi*-xanthatin, was effective against the plant pathogens *Phytophthora drechsleri*, a causal agent of Atractylis rot in pot and field trials (Kim et al., 2002). Since this plant grows abundantly as weed (Bozsa and Oliver, 1990), its utilization for disease control purposes should be advantageous.

Although the isolation of the compound 8-*epi*-xanthatin-1ß, 5ß-epoxide (Joshi et al., 1997; Nour et al., 2009; Saxena et al., 1995; Kim et al., 2003) from *X. strumarium* has been previously reported, but there is no previous report of its antifungal activity.

Table: 3.24. Antifungal activity of 8-*epi*-xanthatine and 8-*epi*-xanthatin-1ß,5ß-epoxide isolated from *Xanthium strumarium*

Tested fungal pathogens	Minimum Inhibitory Concentration (mg/ml)			
	X-1[a]	**X-2**[b]	Bavistin	Nystatin
Plant pathogens	0.039	0.156	0.078	-c
**Colletotrichum gloeosporioides*				
**Colletotrichum camelliae*	<0.0097	0.039	0.078	-
**Curvularia eragrostidis*	0.078	0.625	1.25	-
**Pestalotiopsis theae*	0.078	0.156	0.312	-
**Lasiodiplodia theobromae*	0.039	0.156	0.078	-
**Rhizoctonia solani*	0.0078	0.078	0.039	-
Fusarium equiseti	0.078	0.078	0.156	-
Rhizopus stolonifer	<0.078	0.156	0.039	-
Sclerotinia sclerotum	0.039	0.312	0.078	-
Fusarium oxysporum	0.078	0.078	2.5	-
Human pathogens				
Aspergillus niger	0.312	0.156	-c	0.156
Aspergillus fumigatus	0.019	0.312	-	1.25
Candida albicans	0.312	0.312	-	0.039
Candida tropicalis	0.312	1.25	-	0.625
Trichosporon cutaneum	0.312	0.078	-	0.078
Cryptococcus curvatus	0.019	0.312	-	0.078
Filobasidiella neoformans	0.039	0.625	-	0.019
Mucor circinelloides	0.312	0.312	-	0.156

*Tea pathogens [a] X-1 = 8-*epi*-xanthatin; [b]X-2 = 8-*epi*-xanthatin-1ß,5ß-epoxide, [-c] = not tested.

Thus, this study demonstrates for the first time, the antifungal activity of 8-*epi*-xanthatin-1ß,5ß-epoxide against several fungal pathogens of agricultural and clinical importance. Other activities like antimalarial (Joshi et al., 1997), anti-trypanosoma cruzi and *Leishmania donovani* (Nour et al., 2009) and antitumor activity (Kim et al., 2003) of the compound were reported earlier. Structure–activity relationships studies of these sesquiterpenes have revealed that the activities are mediated by α-methylene-Υ-lactone moeities. In case of antifungal activity, the effectiveness seems to be related to the presence of the unsaturated carbonyl group in the cyclopentene ring (Lavault et al., 2005). The lateral side-chain of 8-*epi*-xanthatin contains an unsaturated ketonic group which may also influence the antifungal activity. On the basis of these structure-activity relationships, it is clear that compounds possessing at least one potentially reactive α,ß-unsaturated carbonyl group are biologically active (Lavault et al., 2005).

3.3.7. Antifungal compound identified in *Piper betle* leaf extracts

3.3.7.1. Chavibetol acetate (1-methoxy-2-acetoxy-4-(2-propenyl) benzene) (PB-1): Compound (**PB-1**) was isolated as yellowish oil from *P. betle* leaf extract after repeated column chromatography. The UV spectrum showed absorption at λ_{max} 228 and 283 nm indicating the presence of monoterpene along with conjugated unsaturation. The compound was spotted at R_f 0.64 when run in TLC showing deep blue colour on spraying with Folin & Ciocalteu's indicating the presence of phenolic group (Table 3.25).

Table: 3.25. Phytochemical properties of compound isolated from *Piper betle* extracts detected by TLC following exposure to UV light and reagent spray

Phytochemical	Type	R_f	UV_{254nm}	Spray reagents		
				Folin & Ciocalteu's	Vanilin-H_2SO_4	Iodine vapour
Chavibetole acetate	Phenolic	0.64	Green	Deep Blue	Brown	Yellow

Table: 3.26. ^{1}H & ^{13}C NMR data of chavibetol acetate isolated from *Piper betle*

Carbon	^{13}C	^{1}H
1	149.3	--
2	139.6	--
3	122.9	6.64-6.67 (m)
4	132.6	--
5	126.6	6.79-6.90 (m)
6	112.3	6.76 (d, *J*=1.5Hz)
7	39.1	3.32 (d, *J*=6.9Hz)
8	137.1	5.86-5.99 (m)
9	115.9	5.04-5.10 (m)
1'	169.0	--
2'	20.6	2.3 (s, 3H)
3'	55.9	3.80 (s, 3H)

IR spectrum showed absorption at 3460, 3076, 3004, 1768 (-O-CO-CH3) and 1639 (C=C) cm^{-1}. The ^{1}H NMR spectrum of **PB-1** displayed several characteristic peaks (Table 3.26). The two very prominent proton singlet at δ_H 2.30 (s) and 3.80 (s), indicated the presence of methyl groups of acetate and methoxy moiety respectively. These two identical peaks of methyl groups were also observed by Evans et al. (1984). The three characteristic aromatic methyne proton signals were observed at range of δ_H 6.64-6.99 (m, 3H, ArH) and an allylic methylene proton at δ_H 5.86-5.99 (m, H-8) on side chain of olefin group. The two methylene proton signals at δ_H 3.32 (d, *J*=6.9Hz, H-7) and 5.04-5.10 (m, H-9) indicated the side chain of olefin group, which was also observed by Ghosh and Bhattacharya (2005). ^{13}C NMR signal and DEPT 90 and DEPT 135 data analysis revealed that **PB-1** (Fig. 3.15) contains four quaternary carbon at δc 169.0 (C-1'), 149.3 (C-1), 139.6 (C-2) and 132.6 (C-4), four methylene signal at 137.1 (C-8), 126.6 (C-5), 122.9 (C-3) and 112.3 (C-6) and two methine signal at 115.9 (C-9) and 39.1 (C-7) ppm. Two characteristic ^{13}C NMR peaks at δc 20.6 (C-2') and 55.9 (C-3') and

their proton singlet at 2.30 (3H, s, $-CH_3$) and 3.80 ppm (3H, s, $-OCH_3$) indicated the presence of methyl group of acetate and methoxy moiety respectively that were directly attached to benzene ring. A ^{13}C NMR signal at 169.0 depicted the occurrence of acetate group. After all spectroscopy data analysis and comparison to previously published data, the final structure was established as chavibetol acetate (1-methoxy-2-acetoxy-4-(2-propenyl) benzene), which was previously reported by Evans et al. (1984).

3.3.8. Bioactivity

Bioautography monitoring of chavibetol acetate (**PB-1**) showed inhibition zones at R_f 0.64 (Table 3.15). The MIC values determined by microtitre plate assay against several tea pathogens along with other plant as well as human pathogens is listed in table 3.27. The compound showed moderate antifungal activity against most of the pathogens. *C. camelliae*, *L. theobromae* and *F. equiseti* were most susceptible among plant pathogens with a MIC value of 0.078 mg/ml. *Filobasidiella neoformans* was the most susceptible human pathogen with an MIC of 0.039 mg/ml.

Chavibetol acetate was previously reported by Evans et al. (1984) with fungicidal and nematocidal activity of five propenylphenols which showed significant fungicidal and nematocidal activity. In another study, chavibetol acetate was found to be a major constituent of *Piper betle* oil. The capillary GC analysis of the oil showed chavibetol (53.1%), chavibetol acetate (15.5%) and several other components. The crude essential oil exhibited antimicrobial activity against *Staphylococcus aureus, E. coli, C. albicans* and *Malassezia pachydermatis*. The strongest activity was observed against *C. albicans*, followed by *S. aureus* and *M. pachydermatis* (Rimando et al., 1986). Row and Ho (2009) studied the chemical compositions of the crude oil by GC/MS analysis and identified 36 compounds reprinting 98.1% of essential oil. Eugenol (36.2%), chavibetol acetate (16.9%), 4-allylphenyl acetate (9.4%) and 4-allylphenol (7.2%) were the main components, comprising 69.7% of the oil.

Table: 3.27. Minimum inhibitory concentration of chavibetol acetate (**PB-1**) isolated from leaves of *Piper betle* against tested fungal pathogens

Tested pathogens	Minimum inhibitory concentration in mg/ml		
	Chavibetol acetate	Bavistin	Nystatin
Plant pathogens			
**Colletotrichum camelliae*	0.078	0.078	-[a]
**Curvularia eragrostidis*	0.156	1.25	-
**Lasiodiplodia theobromae*	0.078	0.078	-
**Pestalotiopsis theae*	0.156	0.312	-
Fusarium equiseti	0.078	0.156	-
Human pathogens			
Aspergillus niger	1.25	-[a]	0.156
Aspergillus fumigatus	0.312	-	1.25
Candida albicans	0.312	-	0.039
Candida tropicalis	0.312	-	0.625
Trichosporon cutaneum	0.312	-	0.078
Cryptococcus curvatus	0.312	-	0.078
Filobasidiella neoformans	0.039	-	0.019
Mucor circinelloides	0.312	-	0.156

[a]'-'= Not tested, * =Tea pathogens

3.3.9. Antifungal compounds identified in *Ocimum sanctum* leaf extracts

3.3.9.1. Eugenol (OS-1): OS-1 was isolated as yellowish oil from the leaves of *O. sanctum* after silica gel column chromatography. The UV spectrum showed absorption at λ_{max} 231 and 282nm indicating the presence of monoterpene with

conjugated unsaturation. IR spectrum showed absorption at ν_{max} 3444 (-OH), 2962, 1608 and 1514 cm^{-1}. The ^{1}H NMR (300MHz, $CDCl_3$) spectrum of compound **OS-1** showed aromatic proton peaks at δ_H 6.66-6.85 (m, 3H aromatic ring), allyl protons at δ_H 5.03-5.10(m, 2H, H-9) and 5.88-6.01(m, 1H, H-8) on side chain of olefin group. Martin et al. (1997) had also reported similar results. The two very prominent proton singlet at δ_H 5.55 (s, -OH) and 3.85 (s, $-OCH_3$), indicated the presence of hydroxyl and methoxy moiety respectively. The ^{13}C NMR spectrum showed characteristic peak of methoxy group at δ_C 56.0 (C-1') and aromatic carbons at δ_C 146.6 (C-1), 144.1 (C-2), 121.3 (C-3), 132.0 (C-4), 114.4 (C-5) and 111.3 (C-6) ppm. The allylic side chain peaks were visible at δ_C 40.0 (C-7), 138.0 (C-8) and 115.6 (C-9) (Table 3.28). After all spectroscopic data analysis, the structure was found to be similar to that of eugenol (Fig. 3.15).

Table: 3.28. ^{1}H & ^{13}C NMR data of eugenol isolated from *Ocimum sanctum*

Carbon	^{13}C NMR	^{1}H NMR
1	146.6	--
2	144.1	--
3	121.3	6.66 – 6.68 (1H, m)
4	132.0	--
5	114.4	6.81 – 6.85 (1H, m)
6	111.3	6.71 – 6.78 (1H, m)
7	40.0	3.31 (2H, d, *J*=6.6)
8	138.0	5.88 – 6.01 (1H, m)
9	115.6	5.03 – 5.10 (2H, m)
1'	56.0	3.85 (3H, s)

3.3.10. Bioactivity

Eugenol was found to be moderately antifungal against most of the tested pathogens of agricultural and clinical importance (Table 3.29). The compound was found to be more effective than bavistin when tested against *C.*

eragrostidis, *P. theae* and *F. equiseti*. Similarly it showed better activity than nystatin against *A. fumigatus* and similar activity against *T. cutaneum.* The most susceptible pathogens producing an MIC of 0.78 mg ml^{-1} were *C. camelliae*, *L. theobromae*, *F. equiseti* and *T. cutaneum*. The compound was least effective against *Candida* sp. showing an MIC of 1.25 mg ml^{-1} against both tested species.

Eugenol is a well known component of several medicinal plants and possess biological activities like antioxidant, cyclooxygenase-linhibitory (Kelm et al., 2000), anthelmintic (Asha et al., 2001), antibacterial (Ali et al., 2005) and antifungal (Kumar et al., 2010). Additionally, eugenol pretreatment prevented liver injury by decreasing CYP2E1 activity, lipid peroxidation indices, protein oxidation and inflammatory markers and by improving the antioxidant status. Single-cell gel electrophoresis revealed that eugenol pretreatment prevented DNA strand break induced by thioacetamide (TA). The increased expression of cyclooxygenase-2 (COX-2) gene induced by TA was also abolished by eugenol (Yogalakshmi et al., 2010).

Antifungal activity has been reported against *Aspergillus flavus* NKDHV8, which cause spoilage of food stuffs during storage and was found to inhibit the aflatoxin production (Kumar et al., 2010). *In vitro* antifungal activity was tested against the medically important yeasts and the MIC and minimal fungicidal concentrations (MFC) were determined as 635 and 293 µg/ml against of *C. albicans* and C*. neoformans* respectively *(*Boonchird and Flegel, 1982*).* Structure-activity relationships could be observed, mainly related to the influence of an allyl substituent at C-4, an OH group at C-1 and an OCH_3 at C-2 (Carrasco et al., 2012). Similar functional groups also present in eugenol and some functional group in chvibetol acetate which showed antifungal activity against phytopathogens and human fungal pathogens. The antifungal activity of eugenol and chavibetol acetate is might be due to the presence of allyl, hydroxyl and methoxy groups which directly attached to benzene ring.

Table: 3.29. Minimum inhibitory concentration of eugenol (**OS-1**) isolated from *Ocimum sanctum* against tested fungal pathogens

Tested pathogens	Minimum inhibitory concentration in mg/ml		
	Eugenol	Bavistin	Nystatin
Plant pathogens			
**Colletotrichum camelliae*	0.078	0.078	-[a]
**Curvularia eragrostidis*	0.156	1.25	-
**Lasiodiplodia theobromae*	0.078	0.078	-
**Pestalotiopsis theae*	0.156	0.312	-
Fusarium equiseti	0.078	0.156	-
Human pathogens			
Aspergillus niger	0.625	-[a]	0.156
Aspergillus fumigatus	0.156	-	1.25
Candida albicans	1.25	-	0.039
Candida tropicalis	1.25	-	0.625
Trichosporon cutaneum	0.078	-	0.078
Cryptococcus curvatus	0.312	-	0.078
Filobasidiella neoformans	0.078	-	0.019
Mucor circinelloides	0.625	-	0.156

[a]'-'= Not tested, * =Tea pathogens

3.3.11. Antifungal compound identified in *Polyalthia longifolia* extracts

3.3.11.1. 16-Oxocleroda-3,13(14)E-dien-15-oic acid (PL-1): 16-Oxocleroda-3,13(14)E-dien-15-oic acid (**PL-1**), a previously known clerodane diterpene was isolated as a colorless semisolid gum compound that was recognized on the basis

of spectroscopy data analysis, when compared to previously reported data (Hara et al., 1995). The molecular formula was determined as $C_{20}H_{30}O_3$ from ESI-MS with an m/z of 318 [M^+]. The UV spectrum showed absorption λ_{max} (Methanol) at 236nm. IR spectral band at λ_{max} 2931cm^{-1}, 1701cm^{-1} and 1643cm^{-1} indicated the presence of alkyl C-H bond, α, ß-unsaturated carbonyl and aldehyde group respectively. The ^{13}C NMR and DEPT spectral data showed a diterpene skeleton with 20 carbons including four methyl carbon δc at 15.9, 18.0, 19.9 and 18.0 are attached at position C-17, C-18, C-19 and C-20 carbon respectively. Six methylene (CH_2) carbon were detected at δ_C 18.1, 26.7, 38.8, 27.5, 37.0 and 19.2 at position C-1, C-2, C-6, C-7, C-11 and C-12 respectively and five quaternary carbons were detected at δc 144.2, 38.2, 39.3, 157.8 and 170.6. Analysis of ^1H and ^{13}C correlation spectral including HMQC indicated the presence of HOOC-CH=C-COH side chain consisting of α, ß-unsaturated carbonyl group (δc 170.2) attached to a branched quaternary carbon (δ_C 157.8) bearing an aldehyde group (δc 194.2 and δ_H 9.5). ^1H NMR and ^{13}C NMR spectral data of **PL-1** (Fig. 3.15) was same with that of the previously reported clerodane diterpene isolated from the stem bark and seeds of *P. longifolia* (Phadnis et al.,1988; Hara et al.,1995). **PL-1** has been reported to be antifeedant (Phadnis et al., 1988), cytotoxic (Chen et al., 2000; Islam et al., 2001) and antimicrobial (Islam et al., 2001; Faizi et al., 2008; Sashidhara et al., 2009). Previous studies on structure activity relation of **PL-1** showed that the free acid group was necessary for its antimicrobial (Faizi et al., 2008) and cytotoxic (Zhao et al., 1991) activities.

3.3.12. Biotransformation of 16-Oxocleroda-3,13(14)E-dien-15-oic acid (PL-1)

Screening level experiments revealed that *R. stolonifer* was capable of rapidly metabolizing compound **PL-1** into polar products in shake cultures. The transformed product was detected in the medium by TLC after incubation for 48 h and onwards along with the substrate. After 4 d, the substrate disappeared from TLC plates and only the product was seen. The uninoculated substrate control or the inoculated control without substrate did not record formation of

this metabolite. To identify the new metabolite, the experiment was repeated in large scale and the compound was extracted after 4 d of incubation from the culture flasks.

3.3.13.18-Hydroxy-16-oxocleroda-3,13(14)E-dien-15-oic acid (PL-2: biotransformed product):

Metabolite **PL-2** (Fig. 3.15) was isolated as a colour less gum from the chloroform extract of the culture media by silica gel column chromatography. The pure compound appeared as a single blue spot on TLC plates upon spraying with anisaldehyde reagent and heating at 110^{o}C which indicated the presence of diterpene. The yield was calculated as 15.6%. The molecular formula was determined as $C_{20}H_{30}O_4$ by ESI-MS and molecular ion peak at m/z 334 [M+] which is 16 amu more than **PL-1**. The strong IR spectral band at λ_{max} 3411, 1697 and 1647 cm^{-1} indicated the presence of hydroxyl, carbonyl and α, ß-unsaturated bond absorption. The ^{1}H and ^{13}C NMR spectral data of **PL-1** and **PL-2** were very similar to each other (Table 3.30). The ^{13}C NMR spectra of **PL-2** showed three-methyl carbon resonating at δc 16.0, 21.4 and 18.2, which were assigned to the C-17, C-19 and C-20 carbon respectively. Seven methylene (CH_2) carbons were resonating at δc 18.2, 26.7, 36.5, 27.4, 37.1, 19.3 and 63.0 is suitable to C-1, C-2, C-6, C-7, C-11, C-12 and C-18 respectively. The ^{1}H NMR and ^{13}C NMR spectral data of **PL-2** showed the loss of a methyl resonating at 18.0 ppm (C-18) as well as the appearance of an allyl signal at δ_C 63.0 (-CH=CH-CH2-) and another new signal at δ_H 4.13. It was therefore clear that hydroxylation had occurred at C-18. Thus, hydroxylation at the allylic methyl group of **PL-1** by biotransformation led to the formation of 18-Hydroxy-16-oxocleroda-3, 13(14) E-dien-15-oic acid (**PL-2**). *R. stolonifer* has been found to catalyze the hydroxylation of the allylic methyl group in diterpenes (Choudhary et al., 2013) and in leucosceptrine, a member of the class of sesterterpene (Choudhary et al., 2006). Allylic hydroxylation has been commonly observed in biotransformation of terpenoids by several fungal systems including *Mucor plumbeus* (Areche et al., 2008),

Glomerella cingulata (Miyazawa and Sugawara, 2005), *Absidia glauca* (Guo et al., 2010) and *Aspergillus niger* (Esmaeili et al., 2012).

A literature study reveals that fungal strains belonging to the genus *Rhizopus* are able to catalyze hydroxylation in a wide range of compounds (Martin, 2010). Phytopathogenic fungi are known to transform antimicrobials to yield multiple products where hydroxylation is the dominant mechanism (Zikmundova et al., 2002; Van Beek et al., 2007) probably involving P450 monooxygenases (Smith et al., 2004).

Table: 3.30. ^{1}H and ^{13}C NMR spectral data of 16-Oxocleroda-3, 13E-dien-15-oic acid (**PL-1**) and 18-Hydroxy-16-oxocleroda-3,13(14)E-dien-15-oic acid (**PL-2**)

Carbon	**PL-1**		**PL-2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	18.1	--	18.2	--
2	26.8	2.05-2.10 (m)	26.7	2.07-2.11 (m)
3	120.7	5.2 (s)	122.7	5.58 (s)
4	144.2 (q_C)	--	147.6	--
5	38.2 (q_C)	--	37.9	--
6	36.8	1.60-1.69 (m)	36.5	1.71-1.81 (m)
7	27.5	1.23-1.28 (m)	27.4	1.24-1.33 (m)
8	36.3	1.58-1.62 (m)	36.5	1.58-1.62 (m)
9	39.3 (q_C)		39.4	
10	46.6	1.41-1.47 (m)	46.5	1.37-1.44 (m)
11	37.0	1.51-1.55 (m)	37.1	1.51-1.56 (m)
12	19.2	2.56-2.60 (m)	19.3	2.52-2.61 (m)
13	157.8 (q_C)	--	156.7	--
14	133.7	6.47 (s)	134.8	6.47 (s)
15	170.6 (q_C)	--	169.1	--
16	194.2	9.55(s)	194.6	9.54 (s)
17	15.9	0.85 (d, 6.6)	16.0	0.85 (d, 6.6)
18	18.0	1.59 (s)	63.0	4.13(s)
19	19.9	0.99 (s)	21.4	1.06 (s)
20	18.0	0.68 (s)	18.2	0.68 (s)

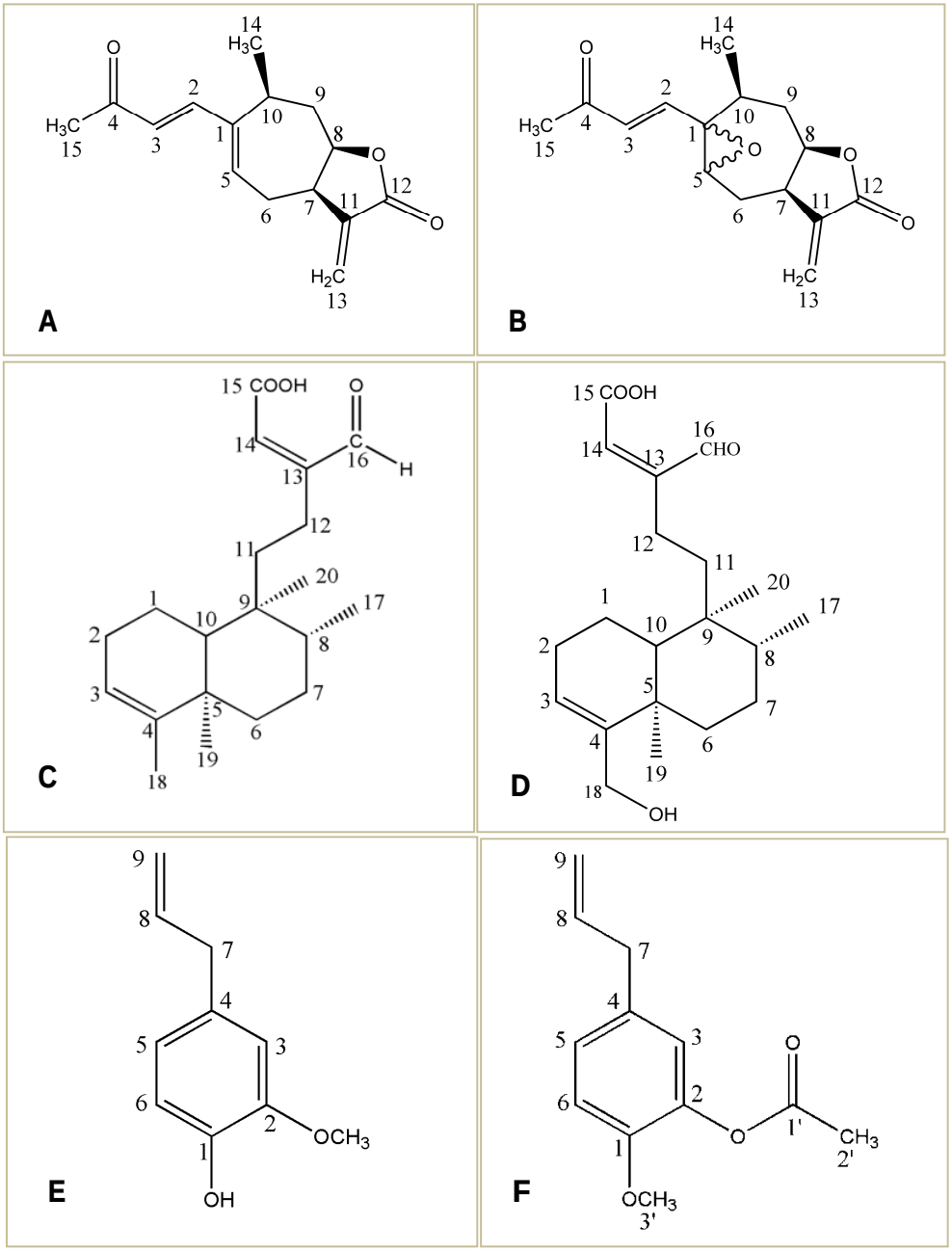

Fig. 3.15. Isolated phytochemical compounds and their sources

A: 8-*epi*-xanthatin and **B:** 8-*epi*-xanthatin-1β,5β-epoxide (*Xanthium strumarium*)

C: 16-Oxocleroda-3, 13(14)E-dien-15-oic acids (*Polyalthia longifolia*)

D: 18-Hydroxy-16-oxocleroda-3, 13(14)E-dien-15-oic acid (Biotransformed products)

E: Eugenol (*Ocimum sanctum*) and **F:** Chavibetol acetate (*Piper betle*)

3.3.14. Bioactivity

The biotransformed compound (**PL-2**) and the isolated parent compound (**PL-1**) were evaluated for their antifungal activity against 13 tested fungal pathogens of clinical and agricultural importance; and activity was compared to the fungicide, nystatin. Altogether eight dilutions (2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 mg ml^{-1}) were obtained by mixing the test compound with potato dextrose broth. MIC values recorded against individual fungi are summarized in table 3.31. **PL-2** was found to possess a higher antifungal activity than **PL-1** against almost all the tested fungi. The lowest MIC (0.039 mg ml^{-1}) of **PL-2** was recorded against *C. eragrostidis*, followed by *A. niger*, *C. gloeosporioides* and *M. circinelloides* each of which were inhibited at an MIC of 0.078 mg ml^{-1}. *A. fumigatus* which was found to be most tolerant to **PL-1** (MIC: 1.25 mg ml^{-1}) showed decreased MIC (0.625 mg ml^{-1}) when tested with **PL-2**. The lowest MIC value of **PL-1** (0.078 mg ml^{-1}) was also recorded against *C. eragrostidis*. It showed moderate inhibition (MIC: 0.156 mg ml^{-1}) against *A. niger*, *C. curvatus*, *C. gloeosporioides*, *L. theobromae* and *M. circinelloides.* The overall results were favourable when compared to nystatin. Thus all tested pathogens apart from *L. theobromae* were more sensitive to **PL-2** than **PL-1**. *L. theobromae* was equally susceptible to both compounds.

There are some literature reports on improvement in antimicrobial activity by biotransformation. For instance, microbial transformation of clerodane lactone, a diterpenoid, by *R. stolonifer*, resulted in the production of several hydroxylated metabolites, some of which showed improved antibacterial activity (Choudhary et al., 2013). Other fungal systems such as *Aspergillus niger* transformed dehydroabietic acid to produce 1*β*,7*β*-dihydroxydehydroabietic acid and 1*β*-hydroxy and 7*β*-hydroxy derivatives which showed weak antimicrobial effect (Gouiric et al., 2004). Capel et al. (2011) used *Mucor rouxii* for biotransformation to produce oleanolic acid derivatives with increased antimicrobial activity against oral bacteria.

Table: 3.31. Minimum inhibitory concentration of 16-Oxocleroda-3,13 (14)E-dien-15-oic acid (**PL-1**) isolated from *Polyalthia longifolia* and **PL-2 (**a biotransformed product of **PL-1**) against tested fungal pathogens

Tested pathogens	Minimum inhibitory concentration (MIC) in mg/ml			
	PL-1	**PL-2**	Bavistin	Nystatin
Plant pathogens	0.156	0.078	0.078	-[a]
**Colletotrichum camelliae*				
**Curvularia eragrostidis*	0.312	0.156	1.25	-
**Lasiodiplodia theobromae*	0.078	0.078	0.078	-
**Pestalotiopsis theae*	0.156	0.078	0.312	-
Fusarium equiseti	0.156	0.078	0.156	-
Human pathogens				
Aspergillus niger	0.156	0.078	-[a]	0.156
Aspergillus fumigatus	>2.5	1.25	-	1.25
Candida albicans	0.312	0.156	-	0.039
Candida tropicalis	>2.5	1.25	-	0.625
Trichosporon cutaneum	1.25	0.625	-	0.078
Cryptococcus curvatus	0.156	<0.156	-	0.078
Filobasidiella neoformans	>2.5	1.25	-	0.019
Mucor circinelloides	0.312	0.156	-	0.156

[a]'-'= Not tested, * =Tea pathogens

In the current study, the transformed compound was detected after 24 h of incubation and the substrate disappeared completely by the 4th day. Further incubation might have resulted in formation of other compounds which could not be done due to low amount of the substrate. To the best of our knowledge this is the first report of isolation of a biotransformed product using fungal system with increased antifungal activity. The increase in polarity by hydroxylation at C-18 probably caused an increase in the permeability of the compound that led to its enhanced bioactivity. However, this increase in antifungal activity was not harmful for the fungus because the product yield was only 15.6%; therefore the concentration in the medium remained well below the MIC.

4.1. Literature review

Ethnobotanical and phytochemical study of plant is important for modern day agriculture but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. A literature review showed that scientists are investigating on the possible utilization of plant products with antimicrobial properties as agricultural fungicide. It would be advantageous not only to standardize methods of extraction or to test the *in vitro* antimicrobial efficacy; but the crude extracts or the discovered compounds should be subjected to *in vivo* testing to evaluate the efficacy in controlling the incidence of disease in crops through pot or field experiments (Gurjar et al., 2012). It is therefore essential to carry out the complete development of an interesting lead compound into an exploitable product.

Although several antifungal extracts from a number of plant species have been noted to be active against a wide range of tea pathogens through *in vitro* tests (Saha et al., 2005a; 2005b), very little work has really focused on development of biorational fungicides in a systematic way to allow interpretation of results and obtain deliverable products that would be applicable in the tea gardens. Considering the enormous need for eco-friendly fungicide in tea gardens, the present work was designed to find out the efficacy of the isolated products as well as the crude extracts in suppressing induced diseases in tea leaves under green-house conditions. A brief literature review reporting the studies with various antifungal plant extracts in controlling plant diseases *in vivo* is presented below.

The efficacy of oily, water-insoluble pastes of *Inula viscosa,* obtained after evaporation of the solvents were tested by Wang et al. (2004) for the control of foliar diseases in growth chambers. The pastes either dissolved in acetone or emulsified in water, effectively controlled downy mildew of cucumber, late blight of potato or tomato, powdery mildew of wheat and rust of sunflower. Mean effective dose (concentration) required for 90% inhibition of disease values for acetone solutions and water emulsion ranged from 0.68 to 1.02% and 0.65 to 1.00% (w/v) respectively. Cohen et al. (2006) reported that the oily paste extract of *Inula viscosa* leaves made with organic solvents were

effective in controlling downy mildew caused by *Plasmopara viticola* in detached leaf tissues of grapes in growth chambers. Four highly effective compounds were detected by thin layer chromatography. Two major inhibitory compounds, each comprising 10.6% of the total paste weight, were identified as tomentosin and costic acid. An emulsified concentrate formulation of the oily paste extracts provided excellent activity against the disease in the field. In whole vines treated and artificially inoculated, the paste concentration required for 90% control of the disease ranged between 0.30 to 0.37%, whereas in naturally infected vines it was 0.58%. It appears that *I. viscosa* is a valuable source for fungicidal preparations against downy mildew of grapes.

Kishore and Pande (2007) evaluated the broad-spectrum antifungal activity of essential oils and their components and their use as fungicides for management of fungal diseases of peanut. Clove oil, cinnamon oil and five essential oil components (citral, eugenol, geraniol, limonene and linalool) were tested for growth inhibition of 14 phytopathogenic fungi. Citral completely inhibited the growth of *Alternaria alternata*, *Aspergillus flavus, Curvularia lunata, Fusarium moniliforme, F. pallidoroseum* and *Phoma sorghina* in paper disc agar diffusion assays. Cinnamon oil, citral and clove oil inhibited the spore germination of *Cercospora arachidicola*, *Phaeoisariopsis personata* and *Puccinia arachidisby* by >90% *in vitro*. The clove oil (1% vol/vol) applied as a foliar spray 10 min before *Phaeoisariopsis personata* inoculation reduced the severity of late leaf spot of peanut up to 58% when challenge inoculated with 10^4 conidia ml^{-1}. This treatment was more effective (P = 0.01) than 0.5% (v/v) citral, cinnamon oil or clove oil and 1% (v/v) eugenol or geraniol.

Choi et al. (2008) reported *in vivo* antifungal activity in methanol extract of *Myristica malabarica* fruit rind which controlled several plant diseases. The methanol extract exhibited potential protective activity against rice blast, tomato late blight, wheat leaf rust and red pepper anthracnose. The authors isolated three antifungal resorcinols from the methanol extract of *M. malabarica* fruit rinds and identified them as malabaricones A (MA), B (MB) and C (MC). Evaluation of inhibitory activity of the three resorcinols against

mycelial growth of plant pathogenic fungi showed variations according to compound and target species. All three compounds effectively reduced the development of rice blast, wheat leaf rust and red pepper anthracnose.

Park et al. (2008) tested *in vivo* fungicidal activity of methanol extracts from 27 medicinal plant species at concentrations of 0.5, 1 and 2 mg/ml against six phytopathogenic fungi causing diseases including rice blast and sheath blight, tomato late blight, cucumber gray mould, barley powdery mildew and wheat leaf rust. Their efficacy varied with plant pathogen, tissue sampled and plant species. At 2 mg/ml concentration, very strong fungicidal activity was produced by extracts of *Boswellia carterii*, *Saussurea lappa*, *Glycyrrhiza uralensis*, *Piper nigrum*, *Rheum coreanum*, *Lysimachia foenum-graecum*, *Evodia officinalis*, *Santalum album* and *Curcuma longa*. At 1 mg/ml, *S. album*, *P. nigrum* and *L. foenum-graecum* showed potent fungicidal activity against *Blumeria graminis* f. sp*. hordei, Puccinia recondite* and *Magnaporthe grisea* respectively. *Lysimachia foenum-graecum* exhibited strong fungicidal activity against *M. grisea* (rice blast) at 0.5 mg/ml.

Al-Reza et al. (2010) reported the efficacy of the essential oil and various organic extracts from flowers of *Cestrum nocturnum* L. for controlling the growth of some important phytopathogenic fungi. The oil (1000 ppm) and the organic extracts (1500µg/disc) showed antifungal effects against *Botrytis cinerea*, *Colletotrichum capsici*, *Fusarium oxysporum*, *Fusarium solani*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorumin*. The growth inhibition ranges were 59.2–80.6% and 46.6–78.9% respectively and their MIC values were ranged from 62.5 to 500 and 125 to 1000µg/ml. The oil displayed remarkable *in vivo* antifungal effect; the disease suppression efficacy ranged between 82.4–100% on greenhouse grown pepper plants.

Yoon et al. (2010) characterized antifungal substances identified as octadeca-9,11,13-triynoic acid and trans-octadec-13-ene-9,11-diynoic acid from the methanol extract of *Prunella vulgaris* and investigated their antifungal activities against the plant pathogens, *Magnaporthe oryzae, R. solani, Phytophthora infestans, S. sclerotiorum, F. oxysporum* f. sp. *raphani* and *P. capsici*. Both compounds showed similar *in vitro* inhibition of mycelial

growth of the plant pathogens. Among the tested pathogens, *P. infestans* was the most sensitive to the two substances with MIC value of 0.84µg/ml. The mycelial growth of *M. oryzae*, *R. solani, F. oxysporum* f. sp. *raphani*, *S. sclerotiorum* and *P. capsici* was moderately inhibited, showing MIC values in a range of 7.6–200µg/ml. *In vivo* antifungal and antioomycete activity of two polyacetylenic acids, suppressed the development of rice blast, tomato late blight and red pepper anthracnose in a dose-dependent manner. They showed weak *in vivo* activity against wheat leaf rust.

Zarafi and Moumoudou (2010) evaluated the *in vitro* and *in vivo* efficacy of cold and hot water extracts of neem leaf and seed, garlic bulb, mahogany seed, ginger rhizome and shea butter leaf in the control of *Curvularia eragrostidis* isolated from pearl millet. Cold water extracts were applied on potted pathogen inoculated pearl millet plants at 2 days before inoculation, 2 days after inoculation and at symptom appearance time. Disease incidence and severity were assessed on these plants. The cold water extract of each tested plant material reduced mycelial growth, sporulation and spore size of the pathogen better than hot water extracts.

Nashwa and Abo-Elyousr (2012) tested antimicrobial activity of six plant extracts that included *Ocimum basilicum* (Sweat Basil), *A. indica* (Neem), *Eucalyptus chamadulonsis* (Eucalyptus), *Datura stramonium* (Jimsonweed), *Nerium oleander* (Oleander) and *Allium sativum* (Garlic) for controlling *Alternaria solani in vitro* and *in vivo*. In *in vitro* study, the leaf extracts of *D. stramonium*, *Azadirachta indica* and *A. sativum* at 5% concentration caused the highest reduction of mycelial growth of *A. solani* (44.4, 43.3 and 42.2% respectively), while *O. basilicum* at 1% and 5% concentration and *N. oleander* at 5% concentration caused the lowest inhibition of mycelial growth of the pathogen. In green house experiments, the highest reduction of disease severity was achieved by the extracts of *A. sativum* at 5% concentration and *D. stramonium* at 1% and 5% concentration. *A. sativum* and *D. stramonium* at 5% concentration increased the fruit yield by 66.7% and 76.2% compared to the infected control. These plant extracts

significantly reduced the early blight disease as well as increased the yield of tomato compared to the infected control under field conditions.

Bajpai and Kang (2012) evaluated the *in vitro* and *in vivo* antifungal efficacy of essential oil and extracts derived from the flower and leaves of *Magnolia liliflora* respectively, against plant pathogenic fungi. The oil (750 µg $disc^{-1}$) and various leaf extracts such as hexane, chloroform, ethyl acetate and methanol (1,500 µg $disc^{-1}$) revealed promising antifungal effects against *Botrytis cinerea*, *Colletotrichum capsici*, *Fusarium oxysporum*, *F. solani*, *Phytophthora capsici*, *R. solani* and *S. Sclerotiorum*. The oil (500µg ml^{-1}) exhibited 100% antifungal effect against leaf spot/scorch of pepper caused by *P. capsici* and at the concentration of 250 µg ml^{-1} had a moderate antifungal effect (83.23%). It was observed that the leaf essential oil displayed potent *in vivo* antifungal effect against one of the selected plant pathogens *P. capsici* on greenhouse-grown pepper plants.

Svecova et al. (2013) reported that *Vitex agnus-castus* methanolic extract showed strong antifungal activity against *Pythium ultimum* in tomato under both *in vitro* and *in vivo* conditions. The 0.2% extract delayed the mycelial growth of the fungus and showed significant antifungal activity against *P. ultimum* on tomato seedlings with an efficacy comparable to that of the synthetic fungicide. To determine the involvement both of plant extract and pathogenic fungus in PR gene induction, tomato plants were treated with *V. agnus-castus* extract and/or inoculated with *P. ultimum*. The expression of four PR genes (PR-1, PR-2, PR-5 and PR-6) was monitored at five time points within 48 h of the extract treatment and fungal inoculation.The activation of various PR genes suggests that induction of defence responses by *V. agnus-castus* extract in tomato may be regulated by more than one signaling pathway.

4.2. Materials and methods

4.2.1. Host plants

Tea plantlets of TV-18 and TV-30 varieties raised from clonal cuttings were obtained from different tea nurseries in the Khoribari, Batasi and Gayaganga tea estate areas located in the Darjeeling district of West Bengal. The collected plantlets were about 20-25 cm in height with 5-10 leaves. These were planted in earthen pots (16cm diameter and 11cm height) containing sterile tea garden soil and maintained in the experimental garden of the Department of Botany, University of North Bengal under normal light and temperature. The plantlets were used for experimental purpose after acclimatization for 30 days.

4.2.2. Preparation of fungal inoculum

The isolated foliar pathogen *Colletotrichum gloeosporioides* strain CR5 causing brown blight and *Curvularia eragrostidis* (ITCC4150.2K) causing leaf spot in tea were used as the test pathogens. The inoculums were prepared either as conidial suspension or as mycelia plugs. The conidial suspension was prepared in sterile distilled water (1×10^6 conidia ml^{-1}) from 10-12 day old PDA cultures (Saha et al., 2005a) as described earlier in section **2.2.5.1.** For preparing mycelial plugs, the fungal pathogen was inoculated in PDA and incubated at 28^oC for five days. A mycelial disc was excised from the advancing zone of fungal hyphae using a sterile cork borer and used for inoculation.

4.2.3. Evaluation of antifungal activity of purified constituent and determination of MIC *in vivo*

Fresh young, fully expanded tea leaves (5-7 cm long) were detached from third or fourth node of mature tea bushes of TV-18 and TV-30 varieties, cleaned thoroughly and placed on blotting paper moistened with sterile distilled water. The blotting paper was laid in trays and covered with a glass lid to form humid chambers. Thirty microlitre of isolated active constituent of phytoextracts (0.025 to 12.8 mg ml^{-1} in ethanol) was placed centrally as spots on the adaxial surface of each leaf on both sides of the midrib. The standard fungicide, bavistin was included in the experiment as positive control. For inoculation,

two wounds (light scratch of 2 mm length) were made with a sterile sharp needle in place of the spots after evaporation of ethanol. Mycelial plugs collected from *C. gloeosporioides* CR5 or *C. eragrostidis* cultures were placed individually on the wounds of the tea leaves and covered with sterile cotton. A set of leaves which received ethanol instead of the purified constituent was considered as untreated-inoculated control. Trays were kept at 28^{o}C in a growth chamber with 12 h photoperiod. The experiment was repeated thrice using ten leaves in each set. Lesion diameters were measured after 24, 48 and 72 h and mean lesion diameter was calculated for each pathogen. The results of three experiments were averaged. The lowest concentration that failed to induce lesion was considered as MIC.

4.2.4. Preparation of crude and purified extract formulations

Fresh leaves of *X. strumarium* and *C. excavata* were collected and washed thoroughly with sterile distilled water and dried at room temperature. The dried leaves were ground separately, weighed and extracted individually with dichloromethane in soxhlet apparatus. The solvent was evaporated at 40^{o}C in a Rotary Vacuum Evaporator. The resulting crude extract (4 mg) and the previously purified compounds (8-*epi*-xanthatin, 8-*epi*-xanthatin-1ß,5ß-epoxide and excavarin-A) (2 mg each) were dissolved separately in 500 µl of acetone and mixed with 40 µl of soyabean oil and 60 µl of Tween 80. Then 900 µl of distilled water was added to each mixture and both were allowed to stand until acetone was evaporated (Wiwattanapatapee et al., 2009). These emulsified extracts were used for testing disease reduction in greenhouse. A separate blank emulsifying mixture was prepared in the same proportions to serve as control.

4.2.5. Control of brown blight and leaf spot of tea by using phytoextracts

For studying the level of protection that may be obtained by the extract preparations, well established young tea plants (7-10 leaves) raised from clonal cuttings of TV-18 and TV-30 varieties were taken in pots (10 cm x 15 cm) containing a mixture of garden soil and farm yard manure (2:1). Experimental plants were sprayed by the formulated extracts on the upper

leaf surface until runoff. Plants sprayed with the blank emulsifying mixture and untreated plants served as control. A fungicide control was included where plants were sprayed with bavistin (2 mg ml^{-1}). The different extracts were sprayed individually at 1 h prior to inoculation with the pathogen. The experimental and control plants were inoculated by spraying a pure conidial suspension (containing 0.05% tween 20) of foliar fungal pathogens (*C. gloeosporioides* CR5 and *C. eragrostidis*) four times at two day interval. Each pathogen was inoculated separately in separate experimental sets. The plants were kept in a transparent and perforated polythene chamber for maintaining high humidity and aeration during the inoculation period. Subsequently the plants were removed from the chamber and grown with normal soil surface watering. Humidity was maintained by spraying sterile distilled water at intervals. The pots were arranged in a randomized block design, with five replicates per treatment. The whole experiment was conducted thrice and the results of individual sets were pooled together and averaged. Assessment for disease development was done after 4, 8, 12, 16 and 20 days of the last inoculation.

4.2.6. Disease evaluation

Assessment of the brown blight and leaf spot disease in both whole plants and detached leaves was done following the method of Dasgupta et al. (2005). The lesions were graded according to their sizes as follows:

Size	grade value
Very small (1–2 mm):	0.1
Small (2–4 mm):	0.25
Medium (4–6 mm):	0.5
Large (>6 mm):	1.0

Number of lesions of each type was counted separately and the value thus obtained was multiplied by its assigned grade value. Disease index of every plant was calculated as the sum total of such values for all its leaves. Results were computed as the mean of observations of all plants in the respective experimental sets and expressed as disease index per plant. Percent efficacy

of disease control (PEDC) was calculated using the formula: PEDC = [(disease index in control – disease index in experimental plants)/disease index in control] × 100 (Purkayastha et al., 2010).

4.2.7. *In vivo* assessment of extract phytotoxicity

In each experiment there was a separate set of treated uninoculated leaves/plants in which only extract was put in drops or sprayed but inoculation was not done, for observing visibility of any tissue necrosis.

4.2.8. Effect of plant extracts on natural bacterial community of the phylloplane and rhizosphere

4.2.8.1. Collection of sample

Healthy leaves of tea and rhizosphere soil were collected from Gayaganga T.E., Happy valley T.E., Margaret Hope T.E., Kamalpur T.E. and N.B.U. campus tea garden. For isolation of bacteria, soil samples which comprised of plant roots with adherent soil were collected in sterilized polythene packets and transported to the laboratory within five hours. Similarly, healthy mature leaves were excised from tea bushes, taken in sterile polystyrene bag and brought separately to the laboratory. The samples from five different locations were tested separately and the mean percentage of bacteria resistant was computed.

4.2.8.2. Isolation of bacteria by serial dilution

For isolation of bacteria from tea rhizosphere, the sample packets of soil were opened and soil adhering to roots was collected by gently shaking the roots. 10 gm of soil sample was mixed in 100 ml of sterile water and kept for 2 hours on a shaker (Aneja, 2003). For isolation from tea phylloplane, each collected leaf was first cut into small pieces (3 mm) and 1 gm of tea leaf pieces was mixed with 10 ml sterile distilled water and kept for 2 hours on a shaker. The resultant sample solutions containing the phylloplane or rhizosphere bacteria were serially diluted separately and bacteria were isolated from each dilution by the spread-plate method. To prepare the dilution series, 1 ml solution was dispensed in another test tube containing 9 ml of sterile distilled water to

make a dilution of 10^{-1}. In similar way, a dilution series of 10^{-1} to 10^{-10} were prepared (Aneja, 2003).

Each different dilution was spread onto solidified agar media to obtain bacterial colonies. To do this, 100µl of each dilution was placed on nutrient agar in petriplates of 9 cm diameter and spread by a glass spreader under aseptic condition. The plates were incubated at 30°C for 48 hours. Each single colony was picked from the agar plates and streaked on sterile nutrient agar plates following quadrant streak method. Pure cultures were maintained on nutrient agar slants at 4°C and sub-cultured at regular intervals.

4.2.8.3. Effect of plant extracts on phyllplane and rhizosphere bacteria of tea plants

Each isolated bacteria was tested *in vitro* for susceptibility towards the formulated crude phytoextracts prepared from *X. strumarium* and *C. excavata*. Nutrient agar medium was autoclaved at 121°C for 15 min, cooled to 45°C and 1 ml of formulated extracts were mixed with 19 ml of molten medium and poured into sterile petriplates of 9 cm diameter. The final concentration of the extract was 2 mg ml^{-1}. After solidification of the medium in petriplates, each of the bacteria isolated from rhizosphere and phylloplane of tea were inoculated in the medium by streaking. Control plates contained nutrient agar amended with blank emulsifying mixture without any phytoextract. The plates were incubated at 30°C and the number of bacteria that grew on the plates was counted after 24 and 48 hours of incubation. The experiment was replicated thrice and the mean number of growing colonies for each location was calculated. The samples from five different locations were tested separately and the mean percentage of bacteria resistant to the phytoextracts was computed.

4.2.9. Statistical analysis

Data were analyzed by ANOVA using Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois. Differences were compared by computing the Critical Difference (CD) at 5% level.

4.3. Results

4.3.1. Evaluation of antifungal activity *in vivo*

The isolated foliar pathogen *C. gloeosporioides* CR5 and leaf spot pathogen *C. eragrostids* were selected for studying the degree of antifungal activity of the compounds isolated from *X. strumarium* and *C. excavata in vivo* in detached tea leaves. The extracted compounds were found to be effective *in vivo* as evident from a progressive reduction in lesion diameter with increasing extract concentration upto 72 h against all the tested pathogens (Figs. 4.1-4.2). MIC values recorded after 72 h are summarized in Table 4.1.

Table 4.1: *In vivo* Minimum inhibitory concentration (MIC) of compounds isolated from *X. strumarium* and C*. excavata* leaves in detached tea leaves of TV-18 and TV-30 varieties against *C. gloeosporioides* CR5 and *C. eragrostidis*

Tested compounds	Minimum inhibitory concentration (mg ml^{-1})			
	C. gloeosporiodes		*C. eragrostidis*	
	TV-18	TV-30	TV-18	TV-30
8-*epi*-xanthatin	0.2	0.1	0.2	0.1
8-*epi*-xanthatin-1ß,5ß-epoxide	0.2	0.2	0.4	0.2
Excavarin-A	0.4	0.2	0.2	0.2
Bavistin	0.4	0.4	>12.8	>12.8

These concentrations were in general found to be lower when tested in detached leaves of TV-30 than in TV-18 variety. 8-*epi*-xanthatin was found to be the best fungicide as it produced an MIC of only 0.1 mg ml^{-1} in TV-30 against both the pathogens followed by 8-*epi*-xanthatin-1ß,5ß-epoxide (0.2 mg ml^{-1} in both TV-18 and TV-30 against *C. gloeosporiodes*). The synthetic fungicide bavistin recorded a MIC value of 0.4 mg ml^{-1} in both TV-18 and TV-30 against *C. gloeosporiodes* but could not control *C. eragrostidis* even at the highest tested concentration. In control leaves which were either untreated or treated with emulsifying mixture and inoculated with the test pathogens, necrotic spots were visible at the wound site after 24 h which gradually increased until 72 h after inoculation. In leaves treated with the purified product at concentrations lower than MIC and inoculated with pathogens,

necrotic spots were visible only after 48 h and the lesion development was slower than untreated leaves. When observed for phytotoxicity of the purified constituent, no necrotic damage was visible in the treated uninoculated leaves.

4.3.2. Control of brown blight and leaf spot in tea

Treatment of tea plantlets with formulations prepared using the compounds purified from *X. strumarium* and *C. excavata* leaf extracts one hour before inoculation with the test pathogens at 2 mg ml^{-1} concentration reduced the disease incidences significantly ($P<0.05$) when compared to control sets (Figs. 4.3-4.6). Severe disease development was noticed in the plants inoculated with the pathogens but not treated with leaf extract or bavistin in both cultivars after 16 days of inoculation. But in plants that were treated with the *X. strumarium* formulation from purified extract one hour prior to pathogens inoculation, brown blight incidence was reduced by 90.4% by 8-*epi*-Xanthatin and by 87.6% by 8-*epi*-xanthatin-1ß,5ß-epoxide in TV-30 variety (Fig. 4.3). Similarly, leaf spot incidence was reduced by 93.3% by 8-*epi*-Xanthatin and by 91.5% by 8-*epi*-xanthatin-1ß,5ß-epoxide in TV-30 variety (Table 4.2).

Table 4.2: Percent efficacy of disease control by purified and crude formulated leaf extracts of *X. strumarium* and *C. excavata* against brown blight and leaf spot of tea

Treatments	Brown blight		Leaf spot	
	TV-18	TV-30	TV-18	TV-30
8-*epi*-Xanthatin	86.2	90.4	92.0	93.3
8-*epi*-xanthatin-1ß,5ß-epoxide	83.1	87.6	89.8	91.5
Crude (*X. strumarium*)	71.1	76.0	74.0	78.3
Crude (*C. excacvata*)	62.6	76.0	35.0	77.5
Excavarin-A	76.0	89.7	53.1	94.3
Bavistin	74.6	84.2	12.4	14.1

The plants treated with crude extract formulation (at 4 mg ml^{-1} concentration) also showed high percentage (76%) of reduction in disease incidence. In case of *C. excavata* extracts, brown blight incidence was reduced by 89.7% by excavarin-A in TV-30 variety (Fig. 4.5) while the crude extract reduced disease

by 76.0%. The purified preparation compared favourably with the synthetic fungicide bavistin whereas the crude extract was slightly less effective.

When observed for phytotoxicity, both pure and crude extract formulation did not show any visible phytotoxicity upto 12 days of spraying. However, after 12 days, plants sprayed only with the crude extract formulation of *X. strumarium* developed light brown spots on leaves at places, where the extract droplets remained adhered. In comparison, the pure compound formulations or the *C. excavata* crude extracts did not show phytotoxicity even after 16 days of spraying.

4.3.3. Effect of plant extracts on phylloplane and rhizosphere bacteria

The nutrient agar plates of lower dilution showed overcrowding of bacteria whereas the higher dilutions produced isolated colonies. In general 10^{-4} to 10^{-6} dilution plates were used for isolation of bacteria from rhizosphere soil samples while 10^{-3} dilution plate was used for the isolation of phylloplane bacteria. Results of susceptibility tests showed that 84% of the bacteria from the phylloplane and 88% of the bacteria from the rhizosphere were resistant to *C. excavata* crude extracts (Fig. 4.7). However, only 62% of the rhizosphere bacteria were resistant towards *X. strumarium* extracts at the tested concentrations.

4.4. Discussion

The importance of botanicals as fungicides in environment friendly agriculture practices has been well documented and reports on the use of phytoextracts to combat plant pathogens are abundant (Dagostin et al., 2010; Tegegne et al., 2008; Yoon et al., 2010; Hafez et al., 2014). Several authors reported the control of fungal diseases by phytochemical components in many crops. For instance, Kishore and Pande (2007) evaluated broad-spectrum antifungal activity of essential oils and their components for control of late leaf spot and crown rot diseases in Peanut. In a study, Bowers and Locke (2000) reported several commercial formulations of botanical extracts and essential oils which were capable of controlling *Fusarium* wilt disease in green house.

Nashwa and Abo-Elyousr (2012) evaluated antifungal activity of various plant extracts against early blight disease of tomato plants under greenhouse and field conditions. The greenhouse and field experiments indicated that the foliar sprays of tomato plants with plant extracts showed a significant reduction in early blight infection.

In order to test of the *in vivo* potential of the purified compounds, 8-*epi*-xanthatin, 8-*epi*-xanthatin-1ß, 5ß-epoxide and excavarin-A were applied against the *C. gloeosporioides* CR5 and *C. eragrostidis* on detached leaves of tea. These purified compounds showed concentration dependent suppression of lesion development in tea leaves. Similar type of disease suppression was shown by *Agapanthus africanus* crude extracts against *Mycosphaerella pinodes* in detached pea leaves (Tegegne et al., 2008). Our results showed that, the *in vivo* MIC ranged from 0.1 to 0.4 mg ml^{-1} against the tested pathogens. It has been further shown that, at this concentration the effect of excavarin-A on phylloplane and rhizosphere microbial community was negligible.

Although the purified compounds were found to be very effective against the tested pathogens, but it is more feasible to use crude extracts for large scale spraying in the field. Therefore we included the crude extract in our greenhouse experiments. Since organic solvents cannot be used for spraying on tea leaves, formulated products were developed by emulsifying the crude and pure extracts in an emulsifying mixture comprising of soybean oil and the surfactant, Tween 80. The final concentration of crude extract was fixed at 4 mg ml^{-1} based on initial preliminary results. Wang et al. (2004) also developed an emulsified product using *Inula viscosa* leaves which effectively reduced several different crop diseases.

All the three tested compounds effectively suppressed tea leaf disease caused by *C. gloeosporioides* and *C. eragrostidis* by both crude and purified extract formulations of *X. strumarium and C. excavata* leaves. Though the medicinal properties of *X. strumarium* and *C. excavata* are well known, they have not been exploited for use as an agricultural product. One report shows that diluted fresh sap obtained from *X. strumarium* plants was highly effective in controlling Atractylis rot caused by *Phytophthora drechsleri* in pot and field

trials (Kim et al., 2002). Since this plant grows abundantly as weed (Bozsa and Oliver, 1990), its utilization for disease control purposes should be advantageous.

In the present study, phytotoxicity was not observable with the purified compounds; however the crude extract of *X. strumarium* was mildly phytotoxic at the tested concentration after 12 days of exposure. However, brown blight and leaf spot could be controlled by 8-*epi*-xanthatin or 8-*epi*-xanthatin-1ß, 5ß-epoxide at much lower concentrations thereby decreasing the chances of phytotoxicity. Carboxyatractylosides found in seeds and seedlings are responsible for the toxicity of *X. strumarium* (Cole et al., 1980; Cutler and Cole, 1983) but adult leaves do not contain this compound (Scherer et al., 2009). Therefore, adult leaves are considered as safe and recommended for use for medicinal purposes (Scherer et al., 2009). Brown blight, grey blight and diplodia disease are listed as the most common diseases prevalent in tea plantations (Singh, 2005).

At present, these diseases are treated by different fungicides including copper compounds which are hazardous for the environment and also have deleterious effect on plants (Saha et al., 2012b). It is therefore necessary that, the chemical fungicides should be replaced by safer arsenals for combating the foliar fungal pathogen. Although there is an urgent need for bio-rational fungicides as more and more tea gardens are shifting to organic production. But, to our knowledge, there are very few reports of botanical products being effective for treating foliar tea diseases (Saha et al., 2005a). In the present study, while the purified compound can be used safely in the present form to control foliar diseases, the crude extract may be used with proper precaution. Considering the high antifungal activity and low phytotoxicity of phytochemicals isolated from *X. strumarium* and *C. excavata* extracts under the test conditions, the extracts may be available for the control of various tea leaf diseases after appropriate field tests. The findings of this study may be significant to the tea industry as an increasing number of tea gardens are now shifting to organic farming methods.

The present work which was undertaken with an aim to utilize phytoextracts for controlling fungal diseases in tea plants, confirmed the presence of several antifungal compounds in five different plant species. The phytochemical compounds, excavarin-A, excavatin-I and osthol purified from *Clausena excavata*; 8-*epi*-xanthatin and 8-*epi*-xanthatin-1β,5β-epoxide purified from *Xanthium strumarium* leaf extracts; 16-Oxocleroda-3,13(14)E-dien-15-oic acid purified from *Polyalthia longifolia*; chavibetol acetate purified from *Piper betle* and eugenol purified from *Ocimum sanctum* showed broad antifungal activity against the tested pathogens of tea. Other compounds such as clausenidin, clausarin, dentatin and nordentatin were also purified along with these antifungal compounds, of which clausarin and nordentatin showed strong antioxidant and DNA damage protective activity. Excavarin-A was reported as a new antifungal coumarin from leaves of *C. excavata* extract. Biotransformation using the fungus *Rhizopus stolonifer* resulted in an increase in antifungal activity of 16-Oxocleroda-3,13 (14)E-dien-15-oic acid due to allylic hydroxylation at C-18 position. Another new compound, 18-hydroxyl,16-oxocleroda-3,13 (14)E-dien-15-oic acid with strong antifungal property was obtained as a result of this derivatization.

Considering the importance of correct identification of plant pathogens, *Exobasidium vexans* causing blister blight and *Colletotrichum gloeosporioides* causing brown blight in tea leaves were identified following DNA based approaches. *C. gloeosporioides* CR5 was isolated from affected tea plants and its pathogenicity was confirmed by verification of Koch's postulations. For detection of *E. vexans*, an unculturable endophyte, part of the 18S rRNA gene of the fungus was amplified by PCR using plant genomic DNA as template which revealed the presence of strains belonging to the genera *Exobasidium*, *Colletotrichum*, *Sporobolomyces*, *Fusarium* and *Neofusicoccum*. Phylogenetic relationship was drawn for *Exobasidium* sp., the causal agent of blister blight, which revealed that this fungus clustered with *E. reticulatum* strains.

Pathogenicity of CR5 was determined in eight clonal varieties of tea by the detached-leaf assay as well as by ELISA. Cross reactive antigens were detected at high levels between pathogen and susceptible tea varieties and at lower levels

when resistant varieties were used. Result showed that TV-18 and TV-30 varieties were more susceptible than other six varieties and were therefore selected for *in-vivo* assays. Emulsified formulations of selected extracts were used for determination of *in-vivo* MIC in detached leaves of tea and for the treatment of tea plantlets made from purified compounds of *X. strumarium* and *C. excavata*. Disease incidence was reduced by 90.4% by 8-*epi*-xanthatin; by 87.6% by 8-*epi*-xanthatin-1ß,5ß-epoxide and by 89.7% by excavarin-A at 2 mg ml^{-1} concentration in TV-30 variety. The results compared favourably with the synthetic fungicide bavistin. The plants treated with crude extract formulation showed 76% reduction in disease incidence. The tested plants extracts showed low toxicity against the rhizosphere and phylloplane bacterial populations.

This study reported the purification of several bioactive compounds effective in controlling foliar fungal diseases of tea. Considering the high antifungal activity and low toxicity of the phytochemicals purified from *X. strumarium* and *C. excavata* extracts under the test conditions, the extracts may be strong candidates for future field tests. The study reveals significant findings that would be beneficial for the tea industry.

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List of major chemicals used

Chemicals	Company
Acetic acid (glacial)	SRL Pvt. Ltd., Mumbai, India
Acetone	SRL Pvt. Ltd., Mumbai, India
Agar powder, Certified	HiMedia Laboratories Ltd, Mumbai, India
Agarose	SRL Pvt. Ltd., Mumbai, India
Aluminium oxide (Neutral) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Ammonium sulphate	HiMedia Laboratories Ltd., Mumbai, India
Anisaldehyde	SRL Pvt. Ltd., Mumbai, India
Ascorbic acid	SRL Pvt. Ltd., Mumbai, India
Barbituric acid	HiMedia Laboratories Ltd, Mumbai, India
Benzene	SRL Pvt. Ltd., Mumbai, India
Bovine albumin fraction-V	HiMedia Laboratories Ltd, Mumbai, India
Calcium chloride, dehydrate, A.R.	HiMedia Laboratories Ltd., Mumbai, India
Calcium phosphate, dibasic, Extrapure	HiMedia Laboratories Ltd., Mumbai, India
Cetyltrimethylammonium bromide, Molecular Biology Grade	Calbiochem, EMD Biosciences, Inc.
Chloroform	SRL Pvt. Ltd., Mumbai, India
Dextrose	HiMedia Laboratories Ltd., Mumbai, India
Diethyl ether	SRL Pvt. Ltd., Mumbai, India
1,1-diphenyl-2-picryl-hydrazil (DPPH)	HiMedia Laboratories Ltd., Mumbai, India
DNA Restriction enzymes	Promega corporation Madison, U.S.A.
dNTP mix (2.5mM each)	Bangalore Genei (India) Pvt. Ltd.
Ethidium bromide	Bangalore Genei (India) Pvt. Ltd.
Ethyl acetate	SRL Pvt. Ltd., Mumbai, India
Ethylenediaminetetra acetic acid disodium salt extrapure A.R.	SRL Pvt. Ltd., Mumbai, India
Ferric chloride	HiMedia Laboratories Ltd., Mumbai, India
Folin Cio calteu's	SRL Pvt. Ltd., Mumbai, India
Freund's complete adjuvant	Bangalore Genei (India) Pvt. Ltd.

Chemicals	Company
Freund's incomplete adjuvant	Bangalore Genei (India) Pvt. Ltd.
Gel loading buffer (6X)	Bangalore Genei (India) Pvt. Ltd.
Goat anti-rabbit IgG-HRP conjugate	Bangalore Genei (India) Pvt. Ltd.
Hexane	SRL Pvt. Ltd., Mumbai, India
Hydrogen peroxide (H_2O_2)	SRL Pvt. Ltd., Mumbai, India
Iodine	HiMedia Laboratories Ltd, Mumbai, India
ITS1	Sigma Aldrich Chemicals Pvt. Ltd., India
ITS4	Sigma Aldrich Chemicals Pvt. Ltd., India
Lactophenol- cotton blue	HiMedia Laboratories Ltd, Mumbai, India
Lipophilic Sephadex	Sigma Aldrich Chemicals Pvt. Ltd., India
Luria Broth	HiMedia Laboratories Ltd., Mumbai, India
Magnesium chloride	HiMedia Laboratories Ltd., Mumbai, India
Methanol A.R.	SRL Pvt. Ltd., Mumbai, India
Methanol for HPLC	SRL Pvt. Ltd., Mumbai, India
Methanol for UV spectroscopy	SRL Pvt. Ltd., Mumbai, India
Nalidixic acid	HiMedia Laboratories, Mumbai, India
Petroleum ether	SRL Pvt. Ltd., Mumbai, India
pGEM T-Easy Vector system (Cloning Kit)	Promega corporation Madison, U.S.A.
Phenol red	HiMedia Laboratories Ltd, Mumbai, India
Potassium ferricyanide	Nice Chemicala Pvt. Ltd. Cochin
Proteinase K	Bangalore Genei (India) Pvt. Ltd.,
PUC 18	Bangalore Genei (India) Pvt. Ltd.,
Quercetin	HiMedia Laboratories Ltd., Mumbai, India
RNase A	Bangalore Genei (India) Pvt. Ltd.,
Silica gel (100-200 mesh) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Silica gel (60-120 mesh) for column chromatography	SRL Pvt. Ltd., Mumbai, India
Silica Gel GF-254 (60-120 mesh)	SRL Pvt. Ltd., Mumbai, India
Sodium carbonate	HiMedia Laboratories Ltd., Mumbai, India

Chemicals	Company
Sodium chloride	Merck Specialities Pvt. Ltd., Mumbai, India
Sodium sulphate	HiMedia Laboratories Ltd., Mumbai, India
Step Up 100bp DNA ladder	Bangalore Genei (India) Pvt. Ltd.
Sucrose	SRL Pvt. Ltd., Mumbai, India
Sulphuric acid	SRL Pvt. Ltd., Mumbai, India
10 X Taq Polymerase buffer E with 15mM $MgCl_2$	Bangalore Genei (India) Pvt. Ltd.
10X Taq buffer A (Tris with 15mM $MgCl_2$)	Bangalore Genei (India) Pvt. Ltd.,
Taq DNA polymerase (3U/µl)	Bangalore Genei (India) Pvt. Ltd.,
TLC Silica gel 60 F_{254} aluminium sheets	Merck, Germany
TMB/H_2O_2 Substrate for ELISA	Bangalore Genei (India) Pvt. Ltd.,
Trichloroacetic acid	Merck, Germany
Tris (Hydroxylmethyl) aminomethane (Tris Buffer, Tris Base) for molecular biology	SRL Pvt. Ltd., Mumbai, India
Tween 20	HiMedia Laboratories Ltd., Mumbai, India
Tween 80	HiMedia Laboratories Ltd., Mumbai, India
Vanillin	SRL Pvt. Ltd., Mumbai, India
Yeast extract powder	HiMedia Laboratories Ltd., Mumbai, India
ß-mercaptoethanol	SRL Pvt. Ltd., Mumbai, India

Chemical fungicides used with their trade name

Trade name	Chemical name
Bavistin	Carbendazim [2-(methoxycarbamoyl)-benzimidazole]
Nystatin	Mycostatin nickel chloride

1. Phosphate buffer (0.05M)

Solution A: 0.1 M solution of Na_2HPO_4 1.42 g

Distilled water 100ml

Solution B: 0.1M solution of NaH_2PO_4 .$2H_2O$ 1.56 g

Distilled water 100ml

Solution-A and solution-B were autoclaved separately and stored at 4^0C.

Mixed 81 ml of solution A with 19 ml of solution B, diluted to 200 ml with distilled water to make final phosphate buffer (0.05M, pH 7.2).

2. Barbital buffer (pH 8.6)

Barbital buffer was prepared by adding solution A to solution B slowly by stirring constantly until the pH of the solution reaches to 8.6.

Solution A: 0.05M Barbituric acid

0.6405 g of barbituric acid dissolves in 100ml of distilled water by constant stirring on a magnetic stirrer.

Solution B: 0.05M Tris base.

1.2114g of Tris base dissolved in 200ml of distilled water.

3. Carbonate buffer (0.1 M), pH 9.6 (coating buffer)

Sodium bicarbonate 10.6 gm dissolved in 100 ml distilled water.

Antigens were diluted in carbonate buffer to make a final concentration of 20µg/ml. Coat the wells of a PVC microtiter plate with the diluted antigens by pipeting, 100µl of the antigen dilution was added in the top wells of the microtiter plate.

4. Phosphate buffered saline (PBS) (0.15M)

Solution A: Na_2HPO_4 (MW 141.96): 4.2588 g

Distilled water 200ml.

Solution B: Na_2HPO_4, $2H_2O$ (MW 156.01): 9.3606 g

Distilled water 400ml.

Mix 140 ml of solution A with 360 ml of solution B and add 4 g NaCl, 100 mg KCl.

5. Phosphate buffered saline -Tween (0.15 M)

The add 0.05% (v/v) Tween 20 in prepared PBS solution.

6. Phosphate buffered saline -1% Bovine serum albumin

Prepared solution (0.15 M PBS) and added 1% BSA.

7. Phosphate buffered saline -Tween+0.5% BSA

Prepared solution PBS-Tween and added 0.5% BSA

8. Sulphuric acid (H_2SO_4) solution (1N)

1 ml concentrated H_2SO_4 mixed with 35 ml distilled water.

9. 50X TAE buffer

Tris base	242 g
0.5 M EDTA (pH 8.0)	100 ml
Glacial acetic acid	57.1 ml
Final volume adjusted	1000 ml

Measured amount all components were completely dissolved by stirring on a magnetic. Final volume was made up to 1000 ml (pH 8.3) and solution was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C.

10. 1X TE buffer

10 mM Tris-HCl (pH 8.0)	5 ml 1M Tris-HCl
1 mM EDTA (pH 8.0)	1 ml 0.5M EDTA

5 ml of 1M Tris-HCl (pH 8.0) and 1 ml of 0.5 M EDTA (pH 8.0) were mixed and adjust the volume up to 500 ml. It was autoclaved to sterilize and stored at 4°C. The pH of Tris-HCl determines the pH of the TE buffer.

11. Preparation of solution-I for competent cell preparation

$MgCl_2.6H_2O$ (80mM)	1.62 g
$CaCl_2.2H_2O$ (20mM)	0.294 g
Distilled water	100ml

Measured amount of magnesium chloride and calcium chloride were dissolved in distilled water. Solution was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C and stored at 4^0C for further use.

12. Preparation of solution-II (100mM) for competent cell preparation

$CaCl_2.2H_2O$	1.46 g
Distilled water	100ml

Measured amount of calcium chloride was dissolved in distilled water. Solution was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C and stored at 4^0C for further use.

13. Preparation of ethidium bromide solution (10 mg ml^{-1})

To prepare 10 ml of 10 mg/ml ethidium bromide, 100 mg of ethidium bromide powder was dissolved in 8 ml water and stirred on a magnetic stirrer for several

hours to dissolve the dye completely. The volume was adjusted up to 10 ml and stored in dark brown bottle.

14. Agarose gel (1.0%)

Agarose powder (1.0 g) was taken in a conical flask and 1X TAE buffer was added to make the final volume to 100 ml. The mixture was then properly boiled, added proper amount of EtBr solution when its temperature is about 55-60°C and poured on a gel casting plate.

15. Agarose gel (1.5%)

Agarose powder (1.5 g) was taken in a conical flask and 1X TAE buffer was added to make the final volume to 100 ml. The mixture was then properly boiled, added proper amount of EtBr solution when its temperature is about 55-60°C and poured on a gel casting plate.

16. IPTG (Isopropylthiogalactoside) solution

1g of isopropylthiogalactoside dissolved in 4ml autoclaved sterile water and store in aliquots at -20^{0}C

17. XGal solution

100 mg of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside dissolved in 5ml of dimethylformamide and stored in glass vial wrapped with aluminium foil at -20^{0}C.

18. Ampicillin

100mg of ampicillin powder was dissolved in 10ml sterile distilled water then 0.2µ filtered, and stored in aliquots at -20^{0}C

19. 2% CTAB in 1M NaCl (100ml) for DNA isolation

CTAB	1.0g
1M NaCl	100ml

Measured amount of CTAB was added to sterile 1M NaCl and heated in water bath to dissolve completely at 60ºC.

20. NaCl (5M) for DNA isolation

NaCl	29.2g
Distilled water	100ml

Measured amount of NaCl was added to distilled water, mixed properly till the salt dissolves and thereafter sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C.

21. EDTA (0.5 mM) (pH 8.0)

Disodium EDTA-dihydrate	18.6g
Distilled water	100ml

Measured amount of disodium EDTA-dihydrate was dissolved in distilled water and mixed vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (~ 10 g of NaOH pellets). Solution was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C.

22. Tris-HCl (1M)

Tris base	12.32 g
Distilled water	100ml

Measured amount of tris base was dissolved in 100 ml of water and pH was adjusted the pH 8.0 with concentrated HCl, made up the final vol to 100 ml. Solution was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C.

23. 70% ethanol for DNA isolation

Absolute ethanol	70 ml
Distilled water	30 ml

24. Potassium ferricyanide solution (1%)

1gm potassium ferricyanide was added in 100ml distilled water and vigorously mixed until potassium ferricyanide completely dissolved.

25. Trichloroacetic acid solution (10%)

10 gm of Trichloroacetic acid was dissolved in 100 ml water to make a solution.

26. $FeCl_3$ solution (0.1%)

1 gm ferric chloride was dissolved in 100ml distilled water

27. 1,1-diphenyl-2-picryl-hydrazil (DPPH) solution (0.2mM)

3.94 mg of DPPH was weighed and dissolved in 50ml methanol to make a solution, fresh each time.

28. Spray reagents

Vanillin –sulphuric acid (VS)

1% ethanol vanillin (solution-I)

10% ethanolic suphuric acid (solution-II)

Anisaldehyde –sulphuric acid (AS)

0.5 ml anisaldehyde was mixed with 10ml glacial acetic acid followed by 85ml methanol and 5ml concentrated sulphuric acid in that order.

Iodine reagent

About 10gm solid iodine sprayed on the bottom of a TLC glass tank; the developed TLC plate is placed into the tank and exposed to iodine vapour.

Folin Ciocalteu

Folin Ciocalteu solution diluted with distilled water 1:1 ratio

29. DNA extraction buffer (5 ml)

Chemicals	Concentration of stock solution	volume in mixture
Tris (pH 8.0)	1M	500µl
NaCl	5M	1.4ml
EDTA (pH 8.0)	0.5M	200µl
B-mercaptoethanol	-	10µl
Distilled water (sterile)	-	2.89ml
CTAB 2% (w/v)	-	100mg

A number of culture media were used during the present work, the names and compositions of these media are given below.

1. Potato Dextrose Broth (PDB)

Peeled Potato	40 g
Dextrose	2g
Distilled water	100ml

The potato was peeled and boiled in double volume distilled water of required amount. The potato broth was filtered through cheese cloth and then required amount of dextrose was added. Finally the medium was sterilized at 15 lb p.s. i. for 15 minutes in autoclave.

2. Potato dextrose agar (PDA)

Peeled potato was boiled and filtered through cheese cloth. The required amount of dextrose and 2% agar powder were added in filtrate. The agar was completely melted by heating the media before sterilization. Finally the medium was sterilized.

3. Oat meal agar (OMA)

Oat meal	4g
Agar agar	1.5g
Distilled water	100ml

Required amount of powdered oat was boiled in distilled water in a water bath, stirred occasionally and strained through cheese cloth. The agar powdered was added to it and melted by heating before the medium was sterilized at 15 lb for 15 minutes.

4. Luria Bertani (LB) broth medium

20g of Luria broth was suspended in 1000ml of distilled water and the medium was heated to dissolve the components completely. The medium was then sterilized by autoclaving at 15 lbs p.s.i. pressure and 121°C for 15 minutes and used as required.

5. Luria Bertani (LB) agar (1.5%) medium

In 1000 ml of LB broth prepared solution, 15 g of agar powder was added and mixed by stirring on a magnetic stirrer. The agar was dissolved by boiling the broth and then sterilized by autoclaving at 15 lbs p.s.i. pressure and 121°C for 15 minutes.

6. Richard's solution / Medium (RM)

KH_2PO_4	5 g
KNO_3	10 g
$FeCl_3$	0.02 g
$MgSO_4$, $7H_2O$	2.5 g
Sucrose	50 g
Distilled water	1000 ml

All the constituents were taken and mixed with required distilled water by stirring and sterilized at 15 lb p.s.i. for 15 minutes.

7. Media preparation for biotransformation

Glucose	10gm
Peptone	5gm
KH_2PO_4	5gm
Yeast extract	5gm
Glycerol	10ml
NaCl	5gm
Distilled water	1000ml

All these constituents were mixed and adjusted at pH 5.5 after that was autoclaved.

Nucleotide sequences of 18S rRNA genes of the foliar fungal diseases which were isolated from infected tea leaves.

1. DWB-1 (*Exobasidium* sp.)

Accession No. JX560484

ATTTCCGTGGTGAACCTGCGGAAGGATCATTATTGAAATTAAAGGGAGATTGCTTTCGAGCATCGC
CCGACACTTTTGATTCTATACACCCGCCGTCGAGATTTATCTTGATGTGCATTTTTTACACACACGA
AATTTTATCAGACTCTTAAAATCAGTCTATAAACTATATACATATAATAAAATAAAACACAACTTTTG
ACAACGGATCTCTTGGTTCTCCCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCAAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCTTGGAGCATGC
CTGTTTGAGTGTCTTGAATATCTCCTCTCATTCATTTTTTAACTAAAGATGGATCGAGAGGGTCCT
TGGGCTTGCATTGGATGTACTTTTATGCTTGCCTTAAATGCATTAGCTGGATTTCAGTAGAGTTGT
TAAGTAACATTGAAAACTCTTGTGATCGAAATCTGCTTCTAATCCATATCTTGATTTCATCGAGATA
TAATTT

2. KW30 (*Exobasidium* sp.)

Accession No. KC493154

TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGCCAAAGTTGAAA
ATTATATCTCGATGAAATCAAGATATGGATTAGAAGCAGATTTCGATCACAAGAGTTTTCAATGTTA
CTTAACAACTCTACTGAAATCCAGCTAATGCATTTAAGGCAAGCATAAAAGTACATCCAATGCAAGC
CCAAGGACCCTCTCGATCCATCTTTAGTTAAAAAATGAATGAGAGGAGATATTCAAGACACTCAAAC
AGGCATGCTCCAAGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTTGATGATTCACTGAATTC
TGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGGGAGAACCAAGAGATCCGTT
GTCAAAAGTTGTGTTTTATTTTATTATATGTATATAGTTTATAGACTGATTTTAAGAGTCTGATAAA
ATTTCGTGTGTGTAAAAAAAATGCACATCAAGATAAATCTCGACGGCGGGTGTATAGAATCAAAAG
TGTCGGGCGATGCTCGAAAGCAATCTCCCTTTAATTTCAATAATGATCCTTCCGCAGGTTCACCTA
CGGAAATCACTAGT

3. Isolated CR5 (*Colletotrichum gloeosporioides*)

Accession No. KC493156

TCCGTAGGTGAACCTGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCTTTGTGAACATACC
TATAACTGTTGCTTCGGCGGGTAGGGTCTCCGCGACCCTCCCGGCCTCCCGCCTCCGGGCGGGTC
GGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATA
ATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATT
CTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTAC
AGCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTTTAC
GTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTTCCAAAGGTTGACCTCGG
ATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

4. OB-1 (*Colletotrichum gloeosporioides*)

Accession No. KC493155

TCCGTAGGTGAACCTGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCTTTGTGAACATACC
TATAACTGTTGCTTCGGCGGGTAGGGTCTCCGTGACCCCCCCGGCCTCCCGCCCCCCGGGCGGGT
CGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAAT
AATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTA
CGGCTGACGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTT
ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTTCCAAAGGTTGACCTC
GGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

5. CC19 (*Colletotrichum gloeosporiodes)*

Accession No. KF836743

TCCGTAGGTGAACCTGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCTTTGTGAACATACC
TATAACTGTTGCTTCGGCGGGTAGGGTCTCCGTGACCCCCCCGGCCTCCCGCCCCCGGGCGGGTC
GGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATA
ATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATT
CTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTAC
GGCTGACGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTA
CGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTTCCAAAGGTTGACCTCG
GATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

6. DW4 (*Colletotrichum gloeosporioides*)

Accession No. KF837644

CCGAGGTCAACCTTTGGAAAATTGGGGGTTTTACGGCAAGAGTCCCTCCGGATCCCAGTGCGAGA
CGTAAAGTTACTACGCAAAGGAGGCTCCGGGAGGGTCCGCCACTACCTTTGAGGGCCTACGTCAG
CCGTAGGGCCCCAACACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCC
AGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTG
ATTATTTGCTTGTACCACTCAGAAGAAACGTCGTTAAATCAGAGTTTGGTTATCCTCCGGCGGGCG
CCGACCCGCCCGGGGGCGGGAGGCCGGGGGGGTCACGGAGACCCTACCCGCCGAAGCAACAGTT
ATAGGTATGTTCACAAAGGGTTGTAGAGCGTAAACTCAGTAATGATCCCTCCGCAGGTTCACCTAC
GGAA

7. DW9 (*Colletotrichum gloeosporioides*)

Accession No. KF836745

CTTTGGAAAATTGGGGGTTTTACGGCAAGAGTCCCTCCGGATCCCAGTGCGAGACGTAAAGTTAC
TACGCAAAGGAGGCTCCGGGAGGGTCCGCCACTACCTTTGAGGGCCTACGTCAGCCGTAGGGCCC
CAACACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGC
GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTGATTATTTGCTT
GTACCACTCAGAAGAAACGTCGTTAAATCAGAGTTTGGTTATCCTCCGGCGGGCGCCGACCCGCCC
GGGGGCGGGAGGCCGGGGGGGTCACGGAGACCCTACCCGCCGAAGCAACAGTTATAGGTATGTT
CACAAAGGGTTGTAGAGCGTAAACTCAGTAATGATCCCTCCGCAGGTTCACCTACGGAA

8. SR7 (*Exobasidium* sp.)

Accession No.KJ400966

GGNTTGATCGGGGGGGCCTTGGTTGGCCGCGTCGAATTCTCGTTCCGCCGCTTATTGATATGCTT
AACTTAGAGGGTAGTCCTACCTGATTTGAGGCCAAACTTGAAAATTATATCTCGATGAAATCAAGA
TATGGATTAGAAGCAGATTTCGATCACAAGAGTTTTCAATGTTACTTAACAACTCTACTGAAATCCA
GCTAATGCATTTAAGGCAAGCATAAAAGTACATCCAATGCAAGCCCAAGGACCCTATCGATCCATC
TTTAGTTAAAAAATGAATGAGAGGAGATATTCAAGACACTCAAACAGGCATGCTCCAAGGAATACC
GAGGAGCGCAAGGTGCGTTCAAAGATTTGATGATTCACTGAATTCTGCAATTCACATTACTTATCG
CATTTCGCTGCGTTCTTCATCGATGGGAGAACCAAGAGATCCGTTGTCAAAAGTTGTGTTTTATTT
TATTATATGTATATAGTTTATAGACTGATTTTAAGAGTCTGATAAAATTTCGTGTGTGTAAAAAAAA
TGCACATCAAGATAAATCTCGACGGCGGGTGTATAGAATCAAAAGTGTCGGGCGATGCTCGAAAG
CAATCTCGCTTTAATTTCAATAATGATCCTTCCGCANGTTCAGCTACGGAAA

9. SR5 (Uncultured fungal endophyte)

Accession No. KJ400968

TCCTCCGCTTATTGATATGCTTAAGNTAGCGAAGTATCCCTACCTGATCCGAGGTCAACCTTAGAA
ATGGGGTTGTTTTACGGCGTAGCCTCCCGAACACCCTTTAGCGAATAGTTTCCACAACGCTTAGGG
GACAGAAGACCCAGCCGGTCGATTTGAGGCACGCGGCGGACCGCGATGCCCAATACCAAGCGAGG
CTTGAGTGGTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGT
TCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA
TCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATTAAGTTTACTCAGACTGC
AAAGTTACGCAAGAGTTTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGGGTCGCCCCGGAG
GCAACAGAGTCGGACAACAAAGGGTTATGAACATCCCGGTGGTTAGACCGGGGTCACTTGTAATG
ATCCCTCCGCAGGTTCACCTACGGAA

10. SR13 (*Sporobolomyces bannaensis*)

Accession No. KJ400967

TTCCGTACGTGAACCTGCGGAAGGAGATTATTGAATAATAGGGTGTCTCATTTAACTGAGAACCCG
ATCTTCCACTTTCTAACCCTGTGCATCTGTTAATGGCGAGCAGTCTTCGGATTGTGAGCCTTCACT
TATAAACACTAGTCTATGAATGTAAACTTTTATAACTATATAAAACTTTCAACAACGGATCTCTTGG
CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCATCTTGCGCTCTCTGGTATTCCGGAGAGCATGTCTGTTTGAGTGTCATG
AATTCTTCAACCCAACTGTTTATTAAAAGCAGATGGTGTTTGGATTCTGAGCGCTGCTGGCCTCGG
CCTAGCTCGTTCGTAATGCACCAGCATCCCTCATACAAGTTTGGATTGACTTGGCGTAATAGACTA
TTCGCTAAGGATTCAGTTTTCGGACTGAGCCAACTTAATGAAGAAAGCTTCTAATCTCAAAGTCTA
CCTTTATTTTAGATCTCAAATCAGGCAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
AA

11. KW19 (*Sporobolomyces* sp.)

Accession No. KJ400972

TAGTCCTACCTGATTTGAGGTCGAATAAAATGGGGTCAGGAGCCTGCGCTTCACGAACAACCAAGC
GAGAGGTCTACAACGCTCGGTCGTCGCGGGCAGCAGAGGCGCTCCGCATGCATTTCGGCCGGGC
CGGCGACCAAGGGCACCGGCCAGAGGCGGCCACGGTCCACAGCCACAGCGAGACAAGCTCGGGG
CGTGAGAGGTTTGCGACACTCAAACAGGCATGCTCTTCGGAATGCCAAAGAGCGCAAGATGCGTT
CAAAGATTCGATGATTCACGTAAATTCTGCAATTCACATTACACTTCGCGTTTCGCTGCGTTCTTCA
TCGATGCGGGAGCCAAGAGATCCGTTGCTGAAAGTTTTGTTGTTTTAGTACGATTTTACATTCGTC
ACTTTTAGAGTAAAAAAAGGTTTAGACCAATTTCACAGGGTAGGGATGAAGTGGGCAGCTCTACGC
GAGCGCCGATTCACTAGTGATCCTTCCGCAGGTTCACCTACGGA

12. KW1 (Uncultured fungal endophyte)

Accession No. KJ400971

TTTAGGGGCAATNCGCCCTCTTCCCGAAATCACCAAAATGGTGCAATAGAGCGTTCAAAGATTCGA
TGATTCACTGTATTCTTCCTTCACATTACTTATCGATTTCGCTGCGTTCTTCATCGATGCNAGAGCC
ATGAGATCCGTTGTTGAAAGTTGTATTTGTTATGATATAATACATTCTTTACACTGATGTTTGTATA
ATATCGCCGGAGCGACTAGATGCACAGGTGTTGAGGATATTAAGTTACCGAAGTAACGTTTAATAA
TGATCCTTCCGCAGGTTCACCTACGGAAATCA

13. RBK 2 (Uncultured fungal endophyte)

Accession No. KJ400970

GCGGCCGCGGGATTCGATTTCCTCCGCTTATTGATATGCTTAAACTCAGCGGGTAATCCTACCTGA
CCTAGGGTTATGATCGGAGCGCCTTGGTAGTTGCGCAATAAAGTCACAACTGGCCTCTCGTGATG
ATTAGGACGCGCATGACACTACGCAACAGTTTGTCAAGCATCACTCGTTGTGACACATCATCCACT
GTGGGGGAATCATTTTTAGGCCAACTGCAGGCGGGAGAGTGCAGGGGCTAACATCCGTCCACAAC
CCTTCGGGGGTAGGGGTG

14. TTRK-3 (*Neofusicoccum parvum*)

Accession No. KJ400973

TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTTGATTCGAGCTCCGGCTCGATCTCCCACCC
AATGTGTACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCCTCCGCACGGCGCCCTTCGGGGGGCT
GGCCAGCGCCCGCCAGAGGACCATAAAACTCCAGTCAGTGAACTTCGCAGTCTGAAAAACAAGTTA
ATAAACTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG
TATTCCGAGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGCCCC
GTCCTCCACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAGAAAACA
CCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTCAAGGTTGACCT
CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

15. TTRK-10 (*Fusarium Solani*)

Accession No. KJ400973

TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTATACAACTCATCAACCCTGTGAACATACC
TAAACGTTGCCTCGGCGGGAACAGACGGCCCCGTGAAACGGGCCGCCCCCGCCAGAGGACCCCCT
AACTCTGTTTCTATAATGTTTCTTCTGAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGAT
CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAG
CGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCCCCCCGTGGGCACA
CGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCG
ACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTCTTCTGAAGTTGACCTCGAATCAGGTA
GGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

16. WBRK 1 (Uncultured fungal endophyte)

Accession No. KJ400969

TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGCCGAGGGTGTAGCCCAGCGTAAAAAACTGG
GCTGCACTCCTTCGGGATTCATCCTTTTGTTGTTATAATCCACGTTTCTTTCCCGGGTTAGGGGAG
GCTGCAAAGCTTCTACCCCCGGGGGAGTACAAACATCTCCTGTTTTTTATTATCGTCTGAAACAAC
TTTTTAAATTAAAATAAAACTACCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATACGTAATGCGAATTGCAGAATTTTGTGAGTCATCGAATTTTTGAACGCACATTGCG
CCCTCTGGTATTCCGGGGGGCATATCTGTTCGAGTGTCATTAGACACCAATCAAGCGATTCATTTC
TGCTTGGTCATGGAGGATGTGGTCACACGCACCTCTTAAATATATGTCGTACTGTCTTCAGTGGTT
CCGTCTACTTCAGATACGGTGTGGTCAAACATGTCGTAATTGAAAGGGATTGTAGAAGTCGGGGC
GTTAATGGTCGCGAGACCTCAACTACAACTCTGGCGCATTGCTAGGTTGACCTCGGATCAGAT

SPECTRAL DATA: Fig. 1 – Fig. 65

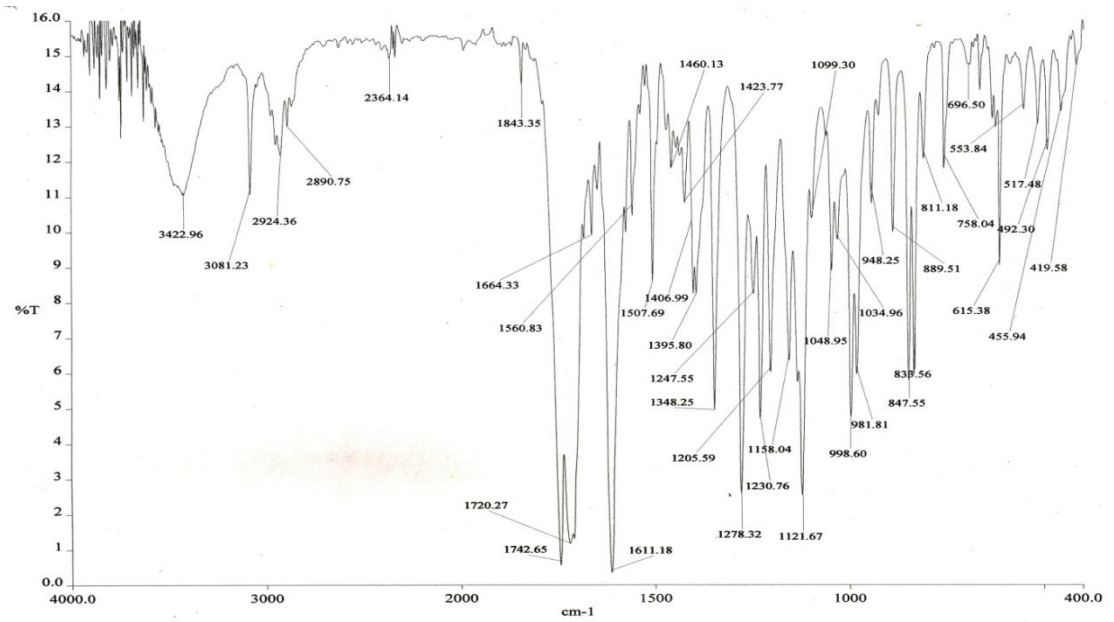


Fig. 1: IR spectral data of Excavarin-A

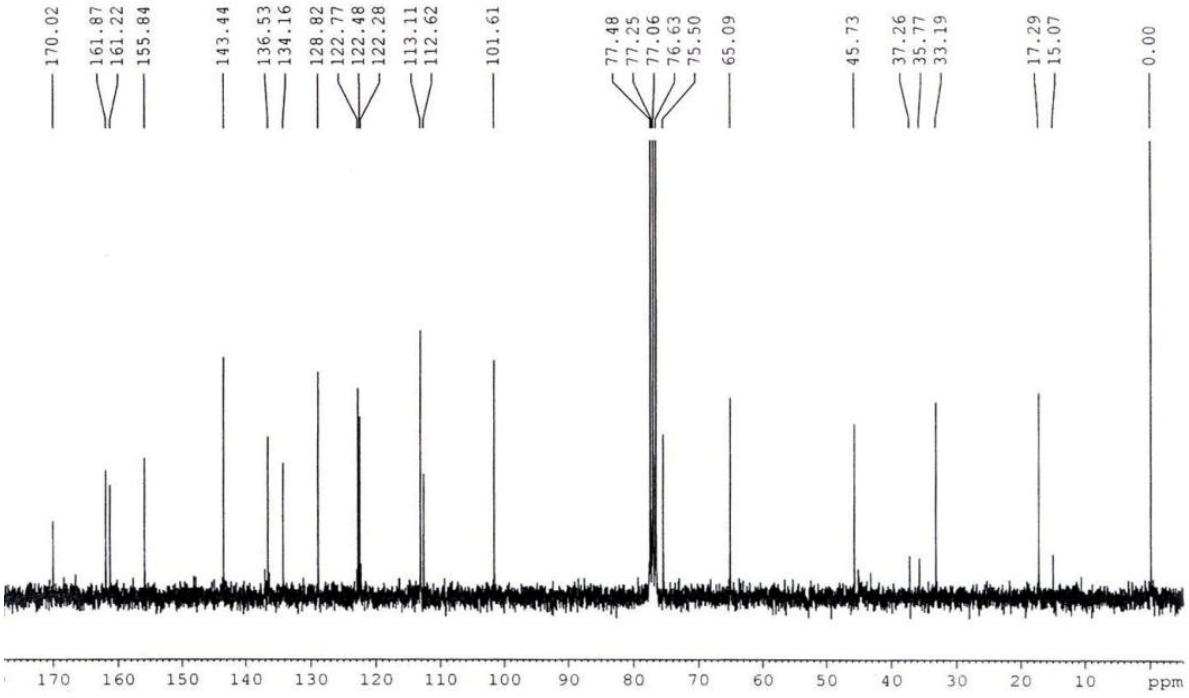


Fig. 2: ^{13}C NMR spectral data of Excavarin-A

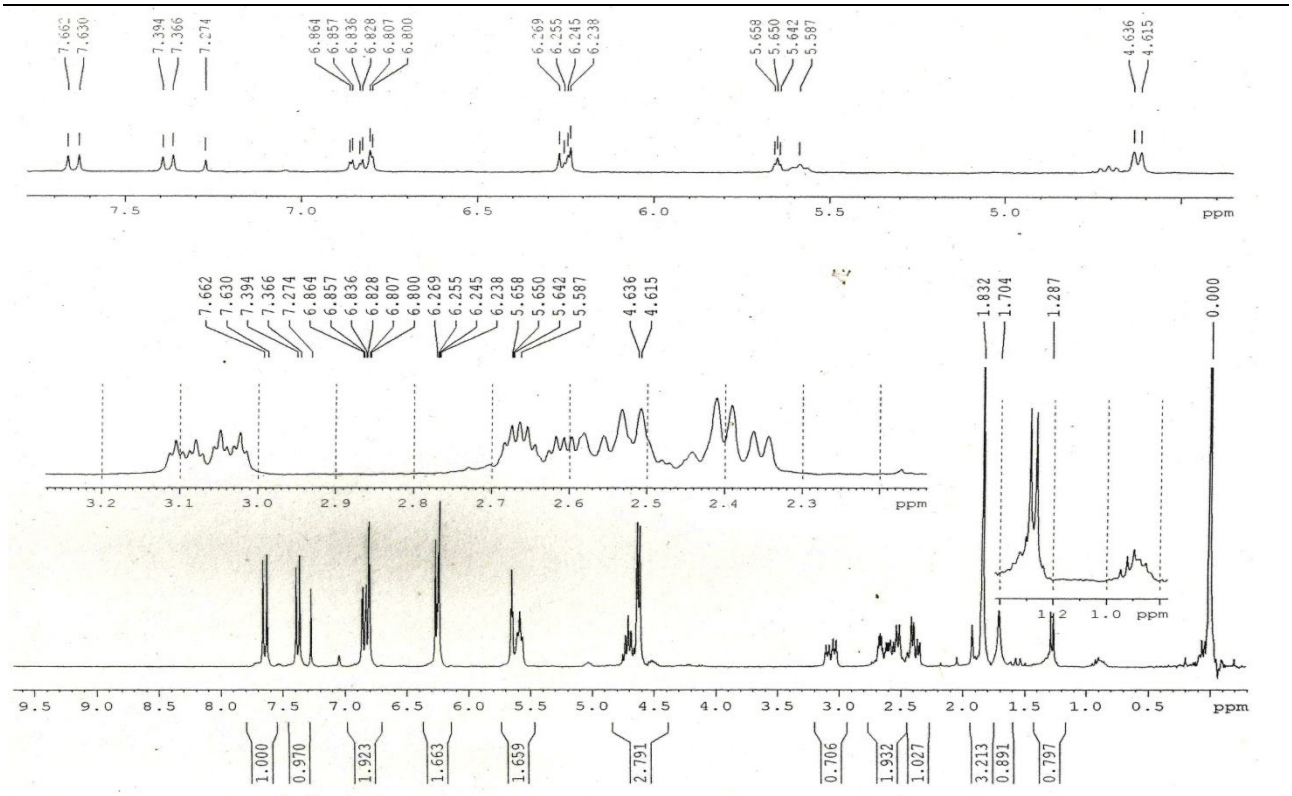


Fig. 3: ^{1}H NMR spectral data of Excavarin-A

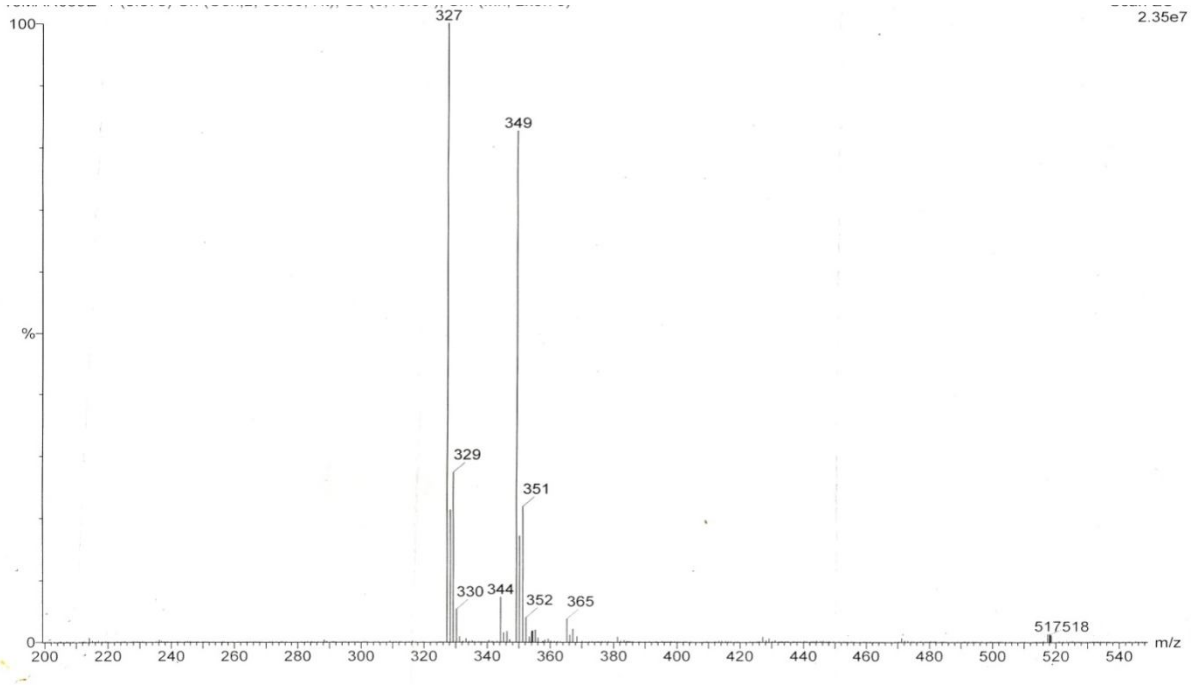


Fig. 4: ESI-MS spectral data of Excavarin-A

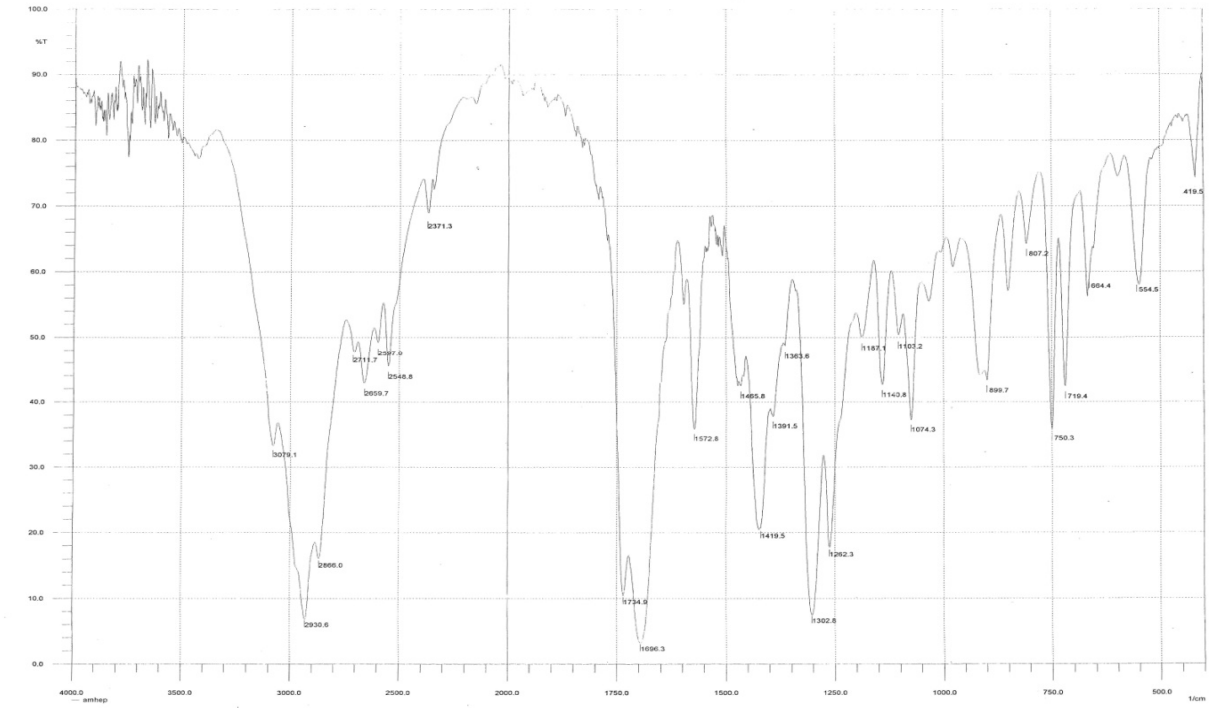


Fig. 5: IR spectral data of Excavatin-I

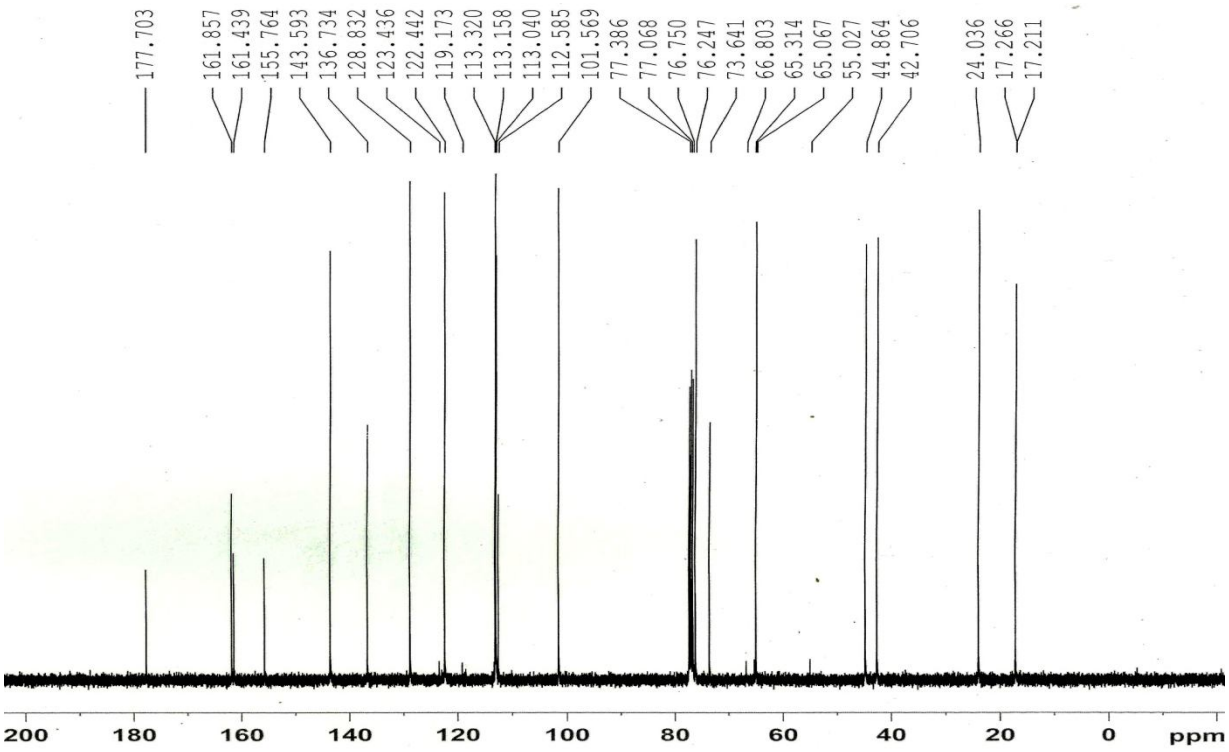


Fig. 6: ^{13}C NMR spectral data of Excavatin-I

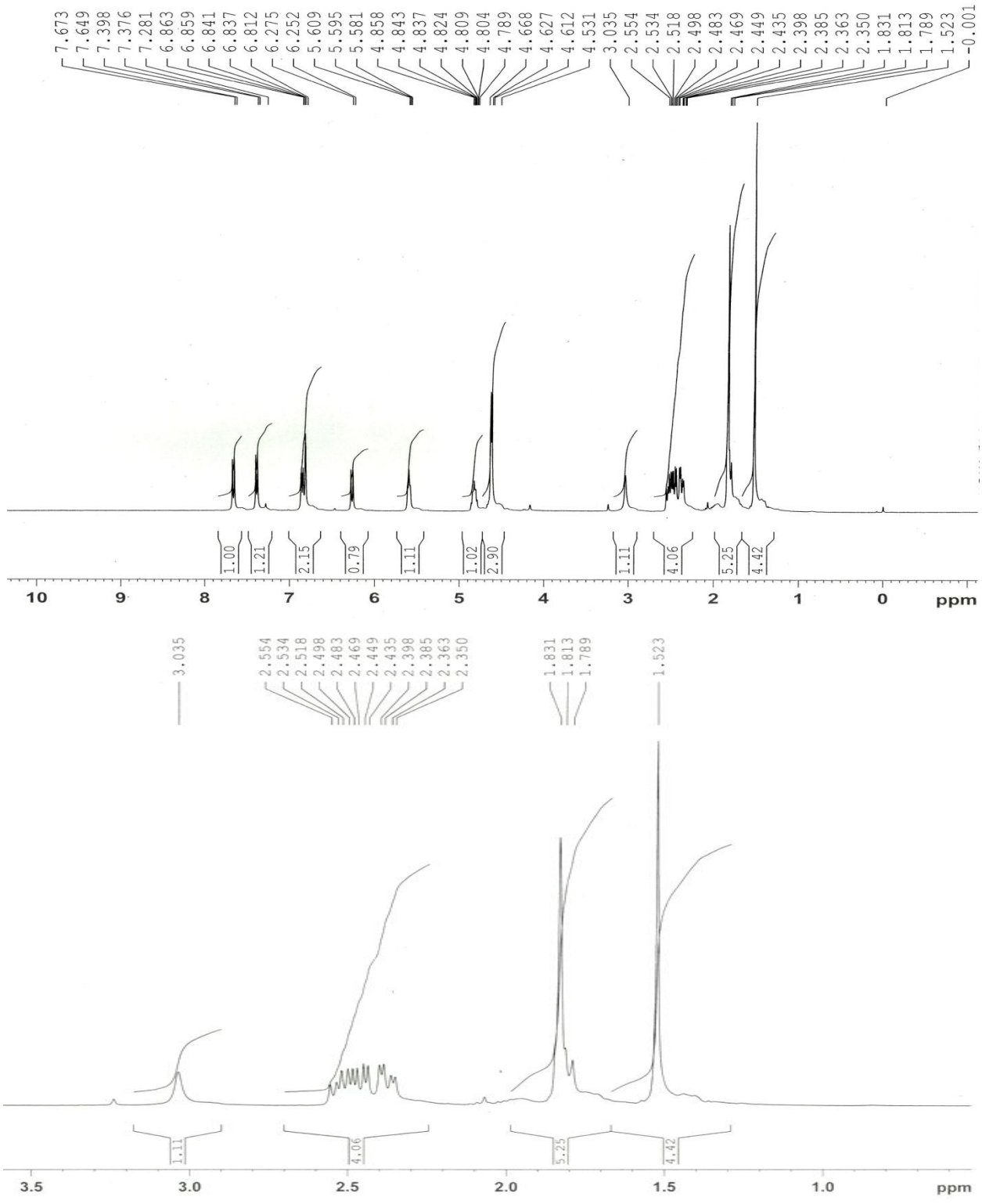


Fig. 7: ^{1}H NMR spectral data of Excavatin-I

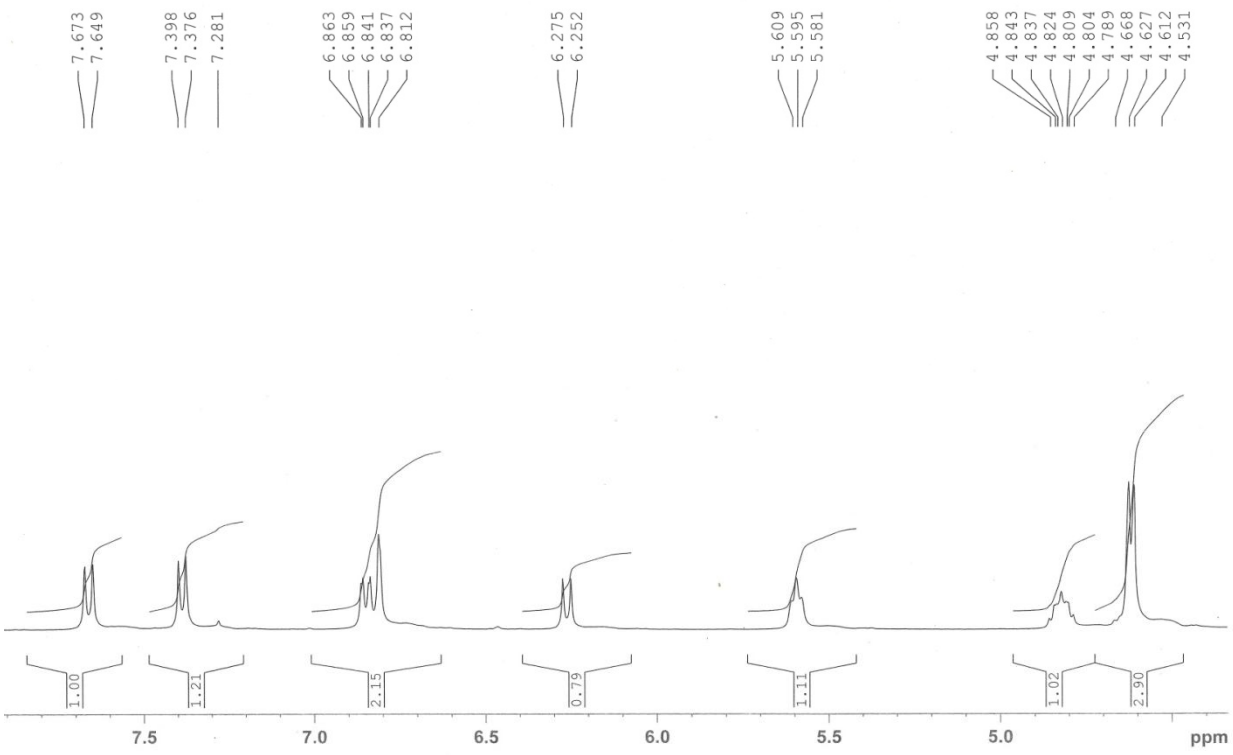


Fig. 8: ^{1}H-NMR spectral data of Excavatin-I

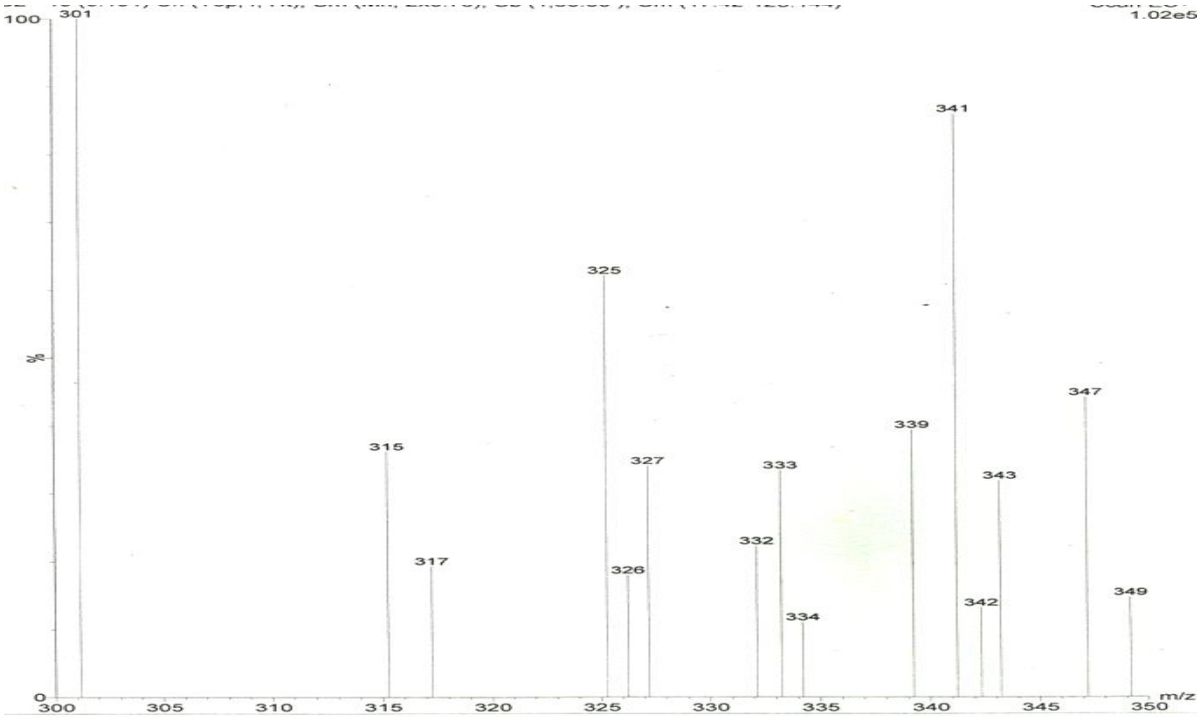


Fig. 9: ESI-MS spectral data of Excavatin-I

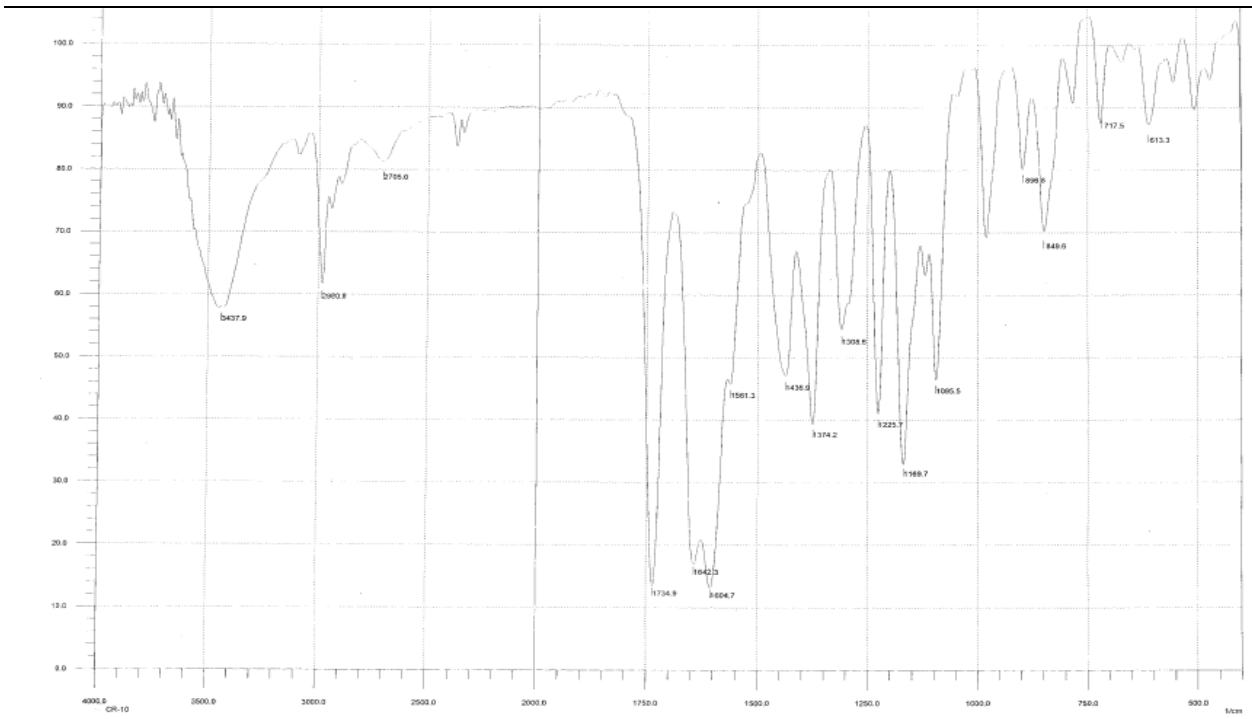


Fig. 10: IR spectral data of Clausenidin

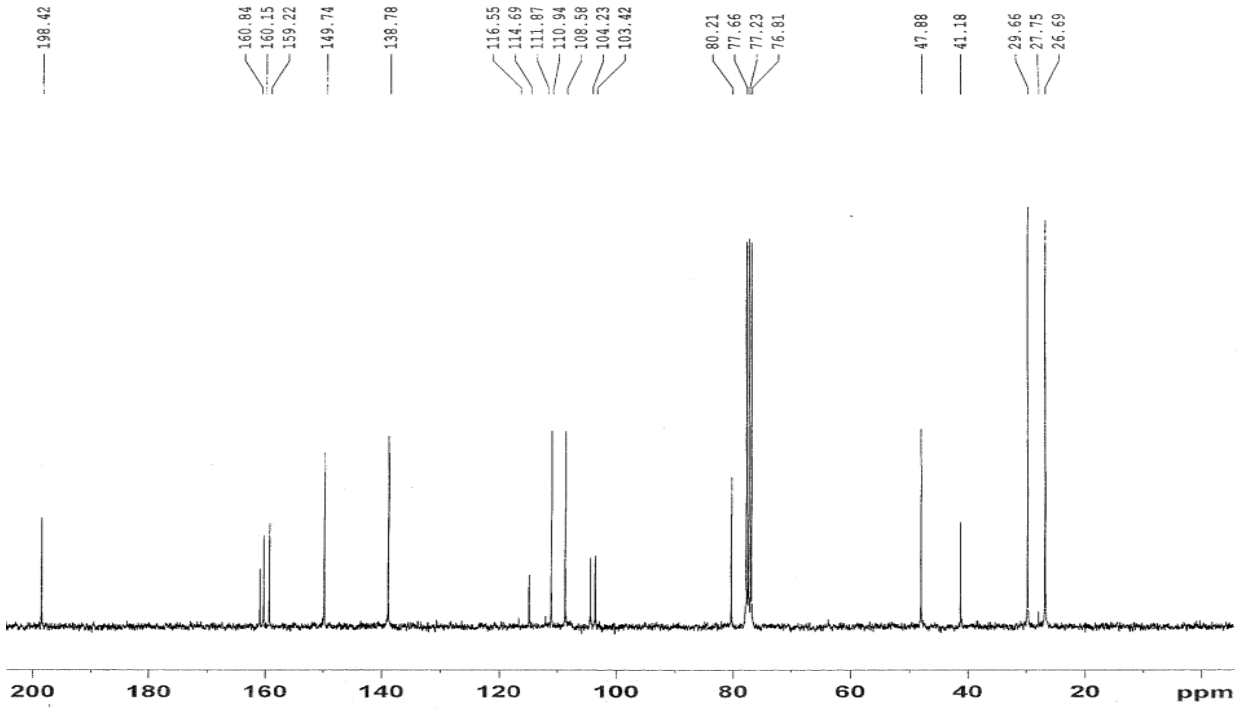


Fig. 11: ^{13}C NMR spectral data of Clausenidin

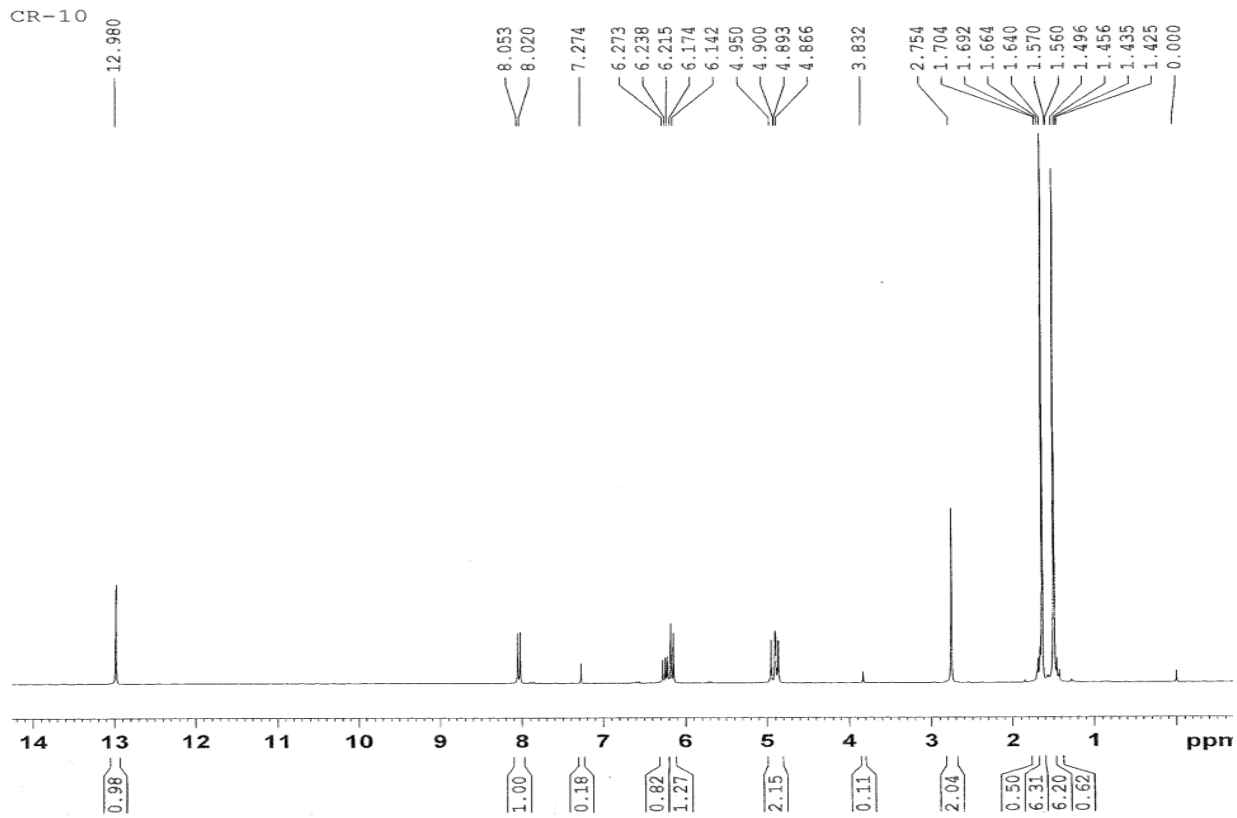


Fig. 12: ^{1}H NMR spectral data of Clausenidin

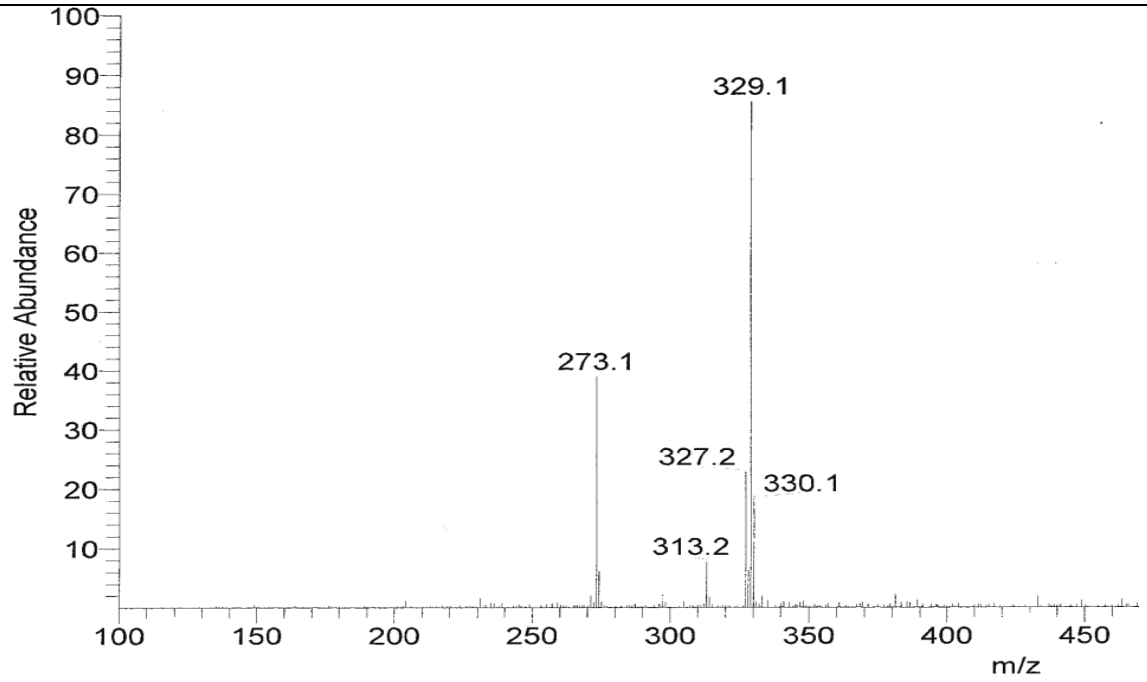


Fig. 13: ESI-MS spectral data of Clausenidin

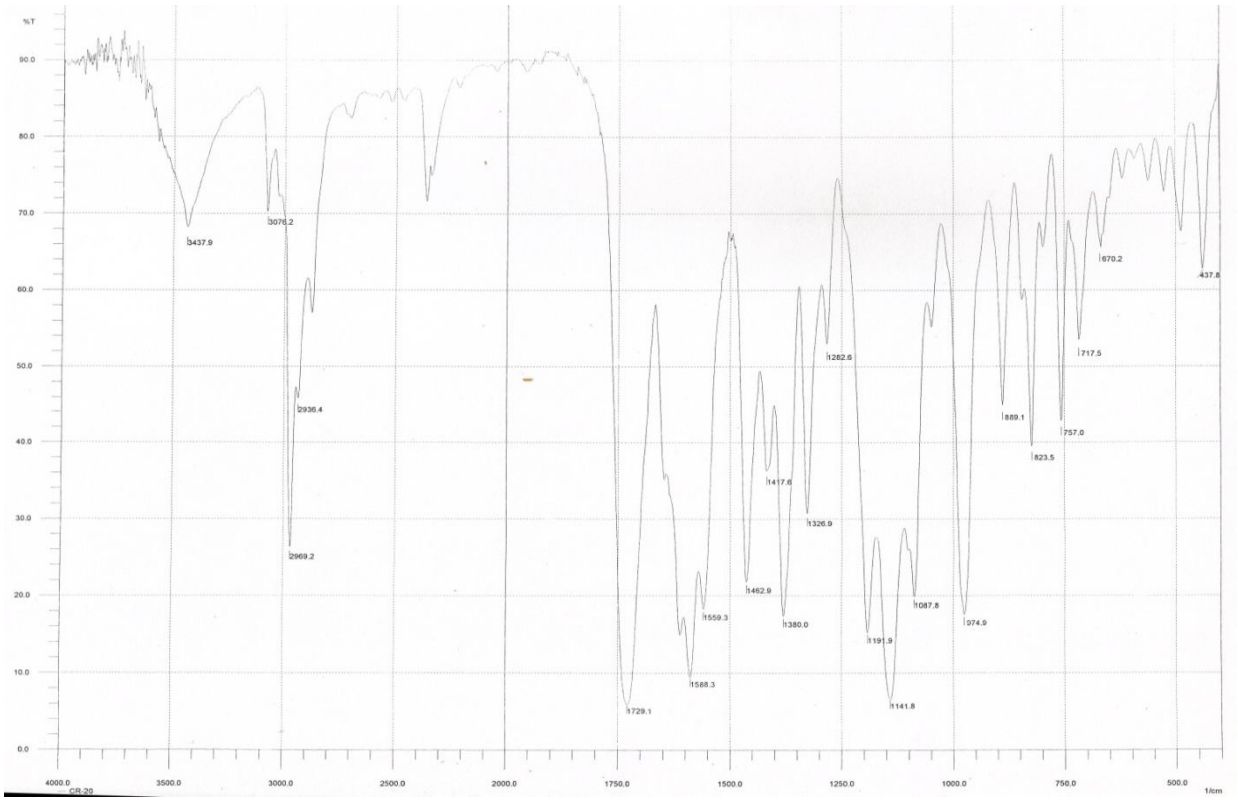


Fig. 14: IR spectral data of Dentatin

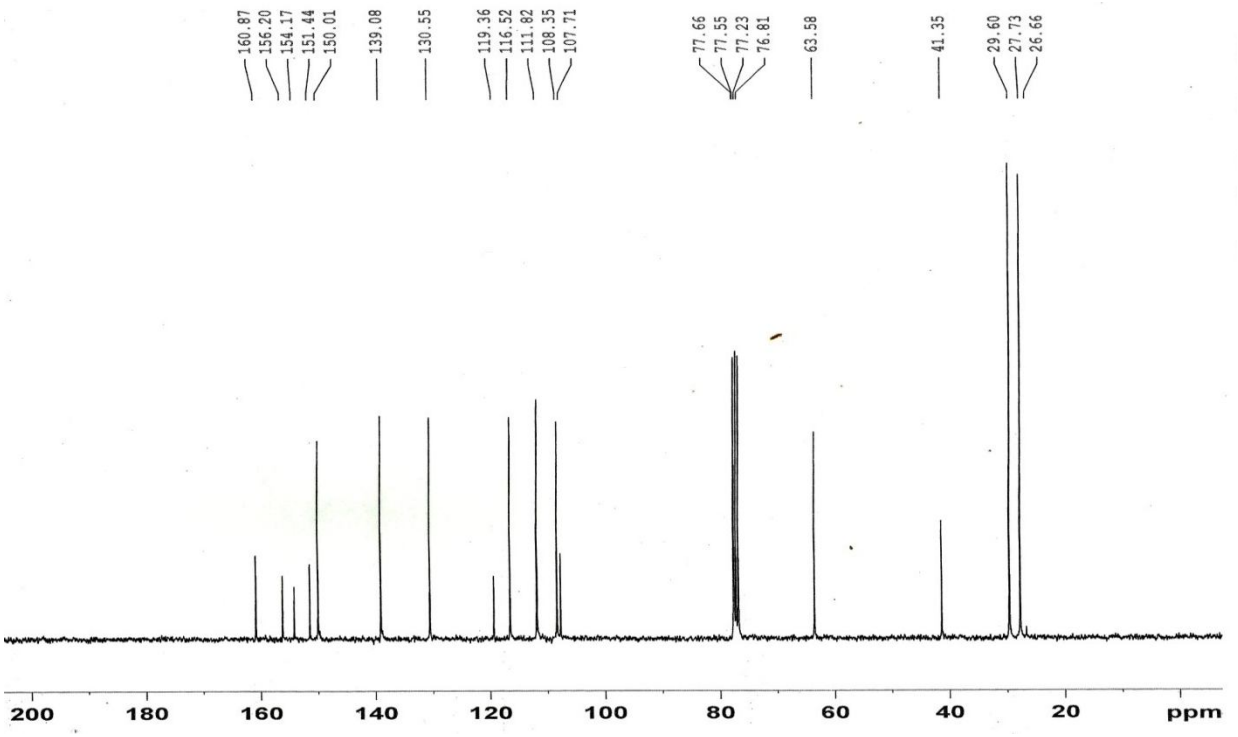


Fig. 15: ^{13}C NMR spectral data of Dentatin

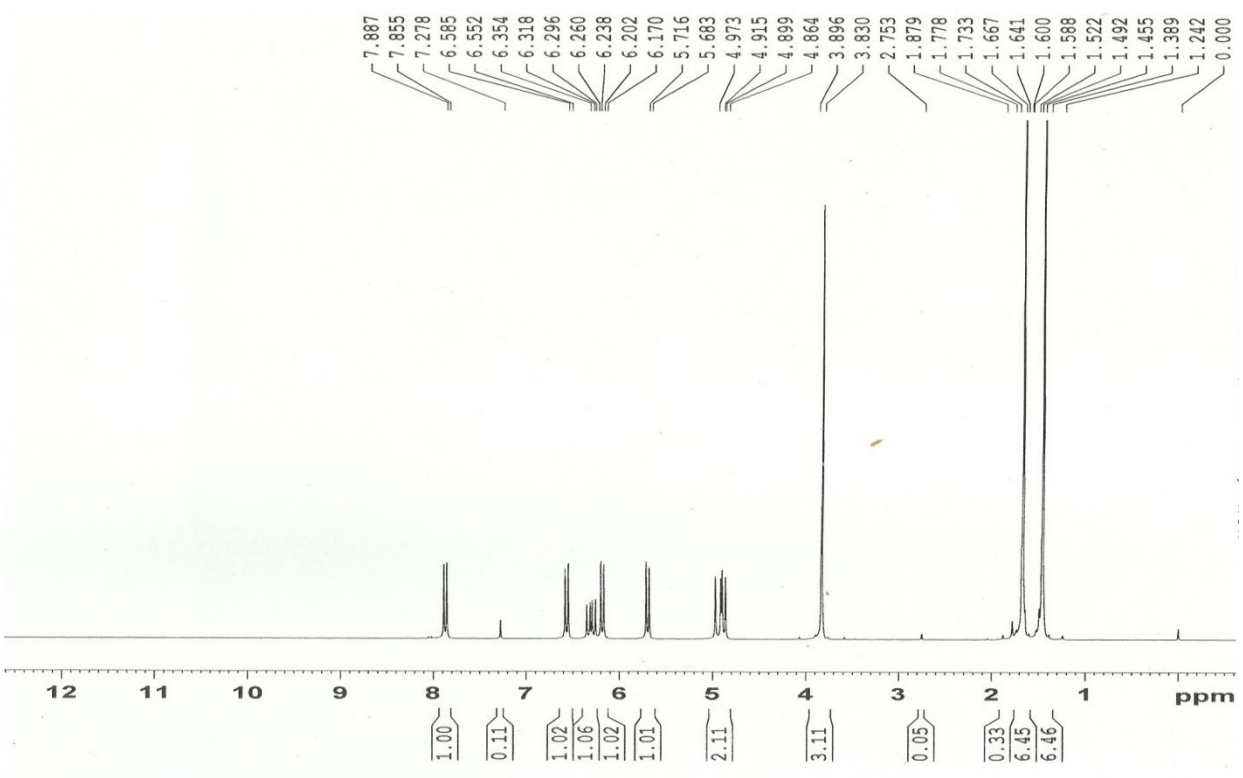


Fig. 16: [1]HNMR spectral data of Dentatin

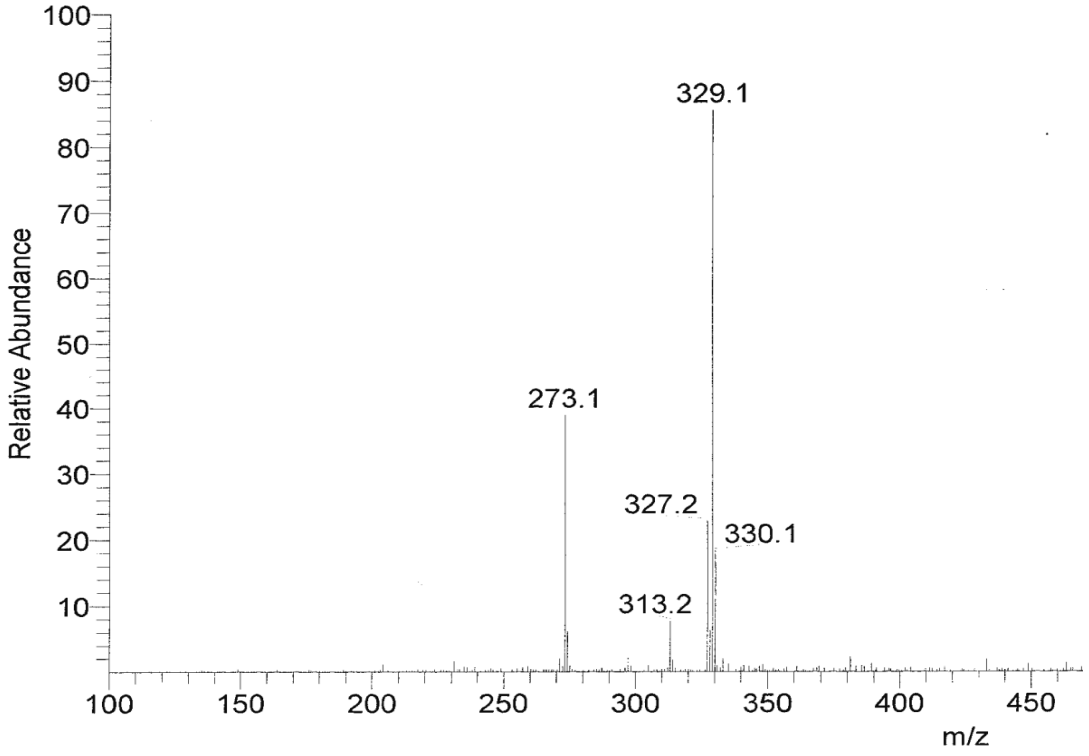


Fig. 17: ESI-MS spectral data of Dentatin

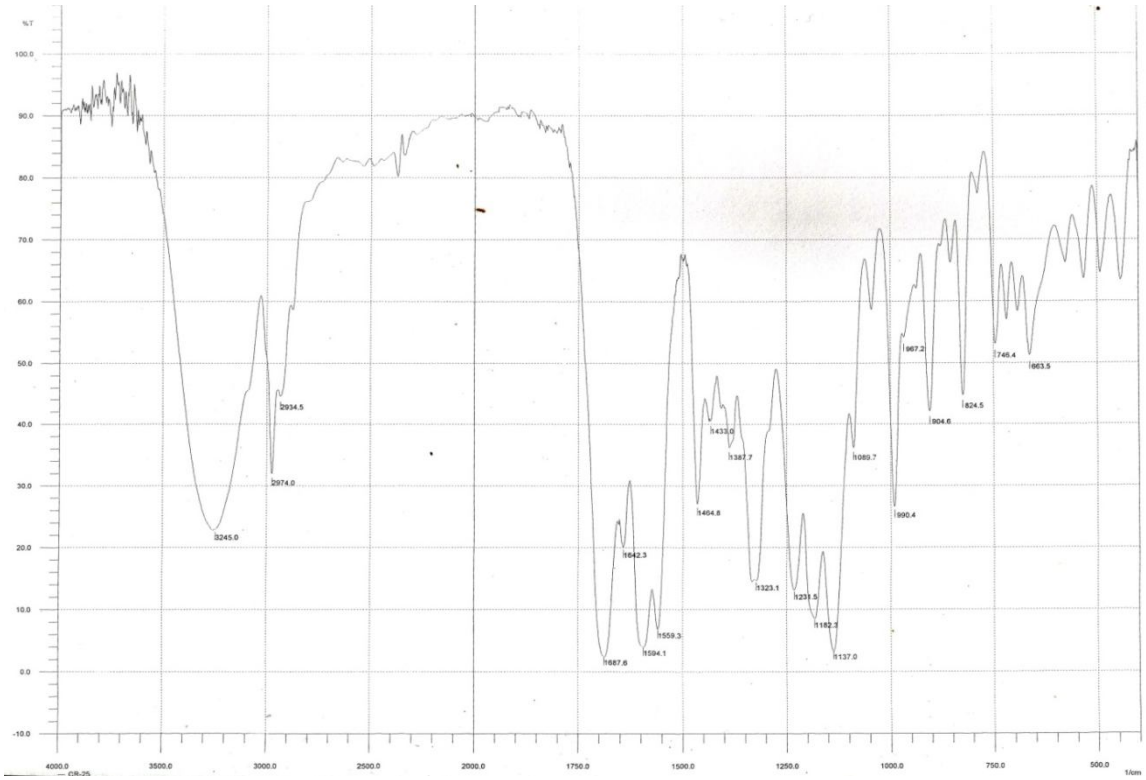


Fig.18: IR Spectral data of Nordentatin

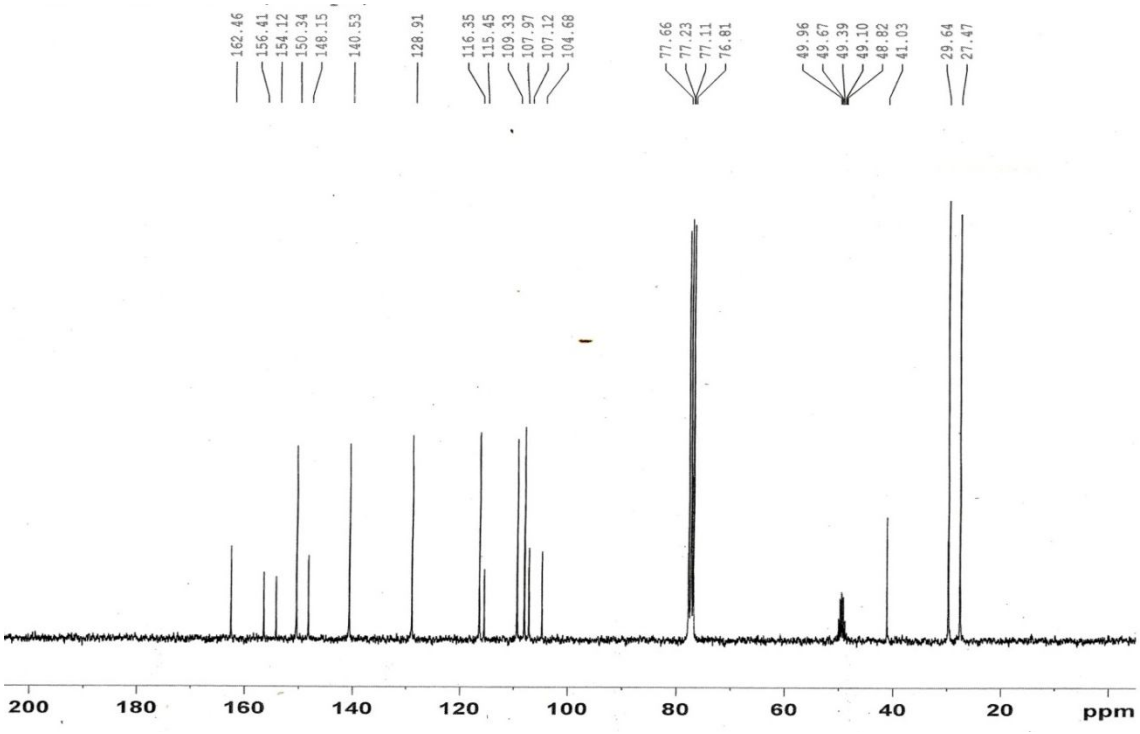


Fig. 19: ^{13}C NMR spectral data of Nordentatin

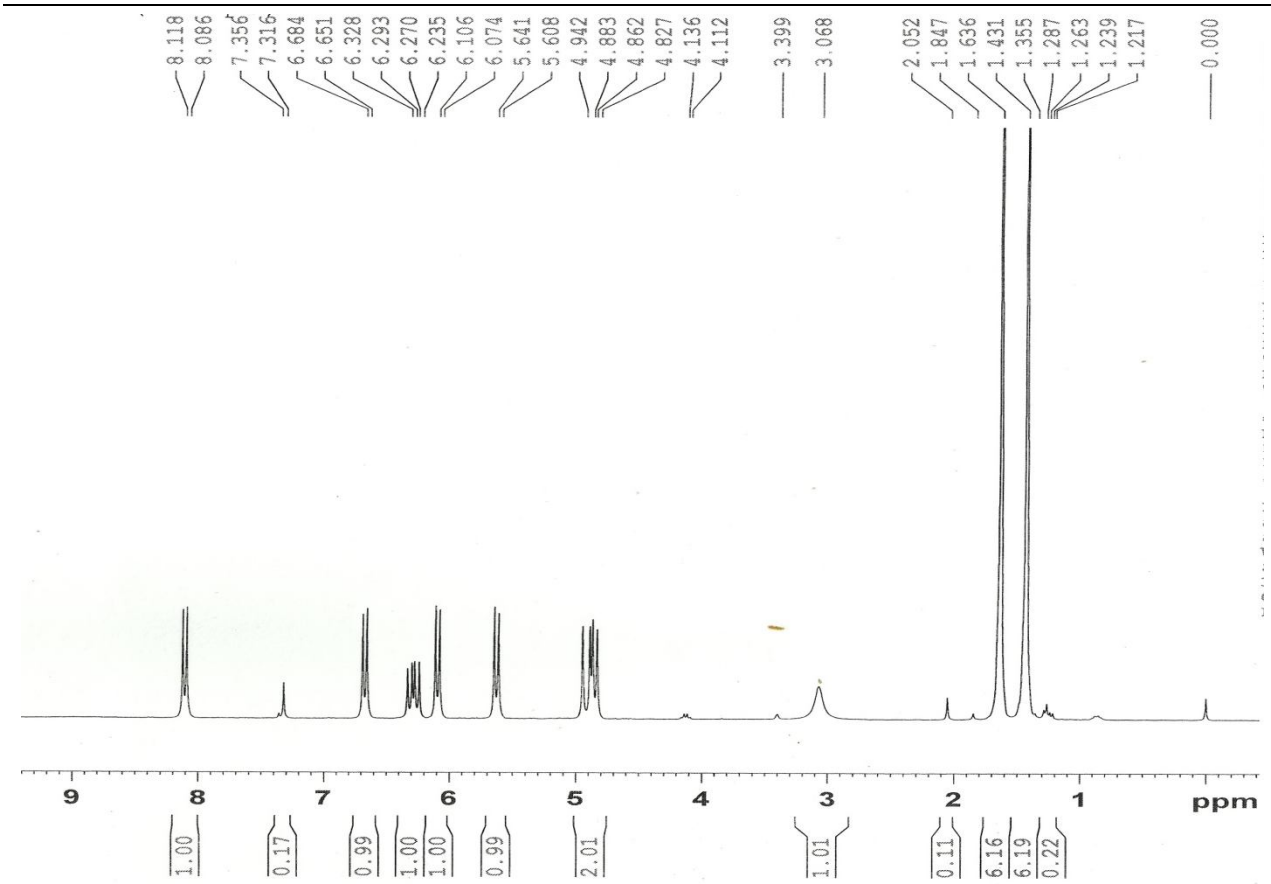


Fig. 20: ^{1}H NMR spectral data of Nordentatin

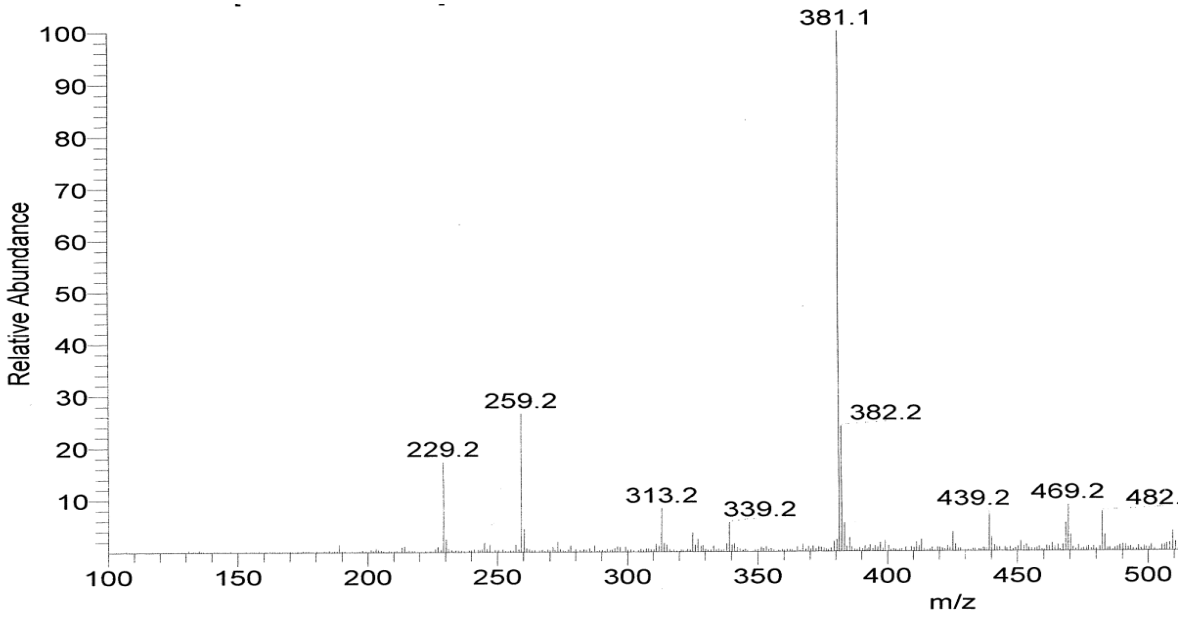


Fig. 21: ESI-MS spectral data of Nordentatin

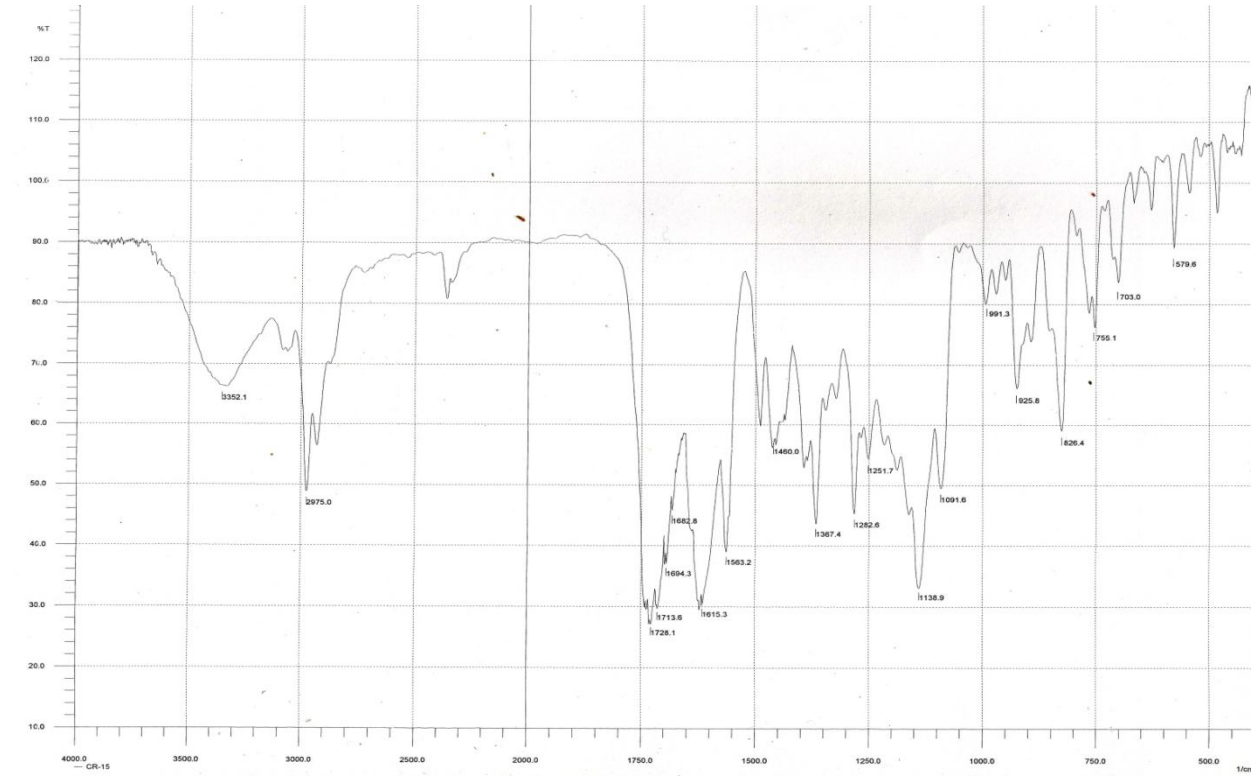


Fig. 22: IR Spectral data of Osthol

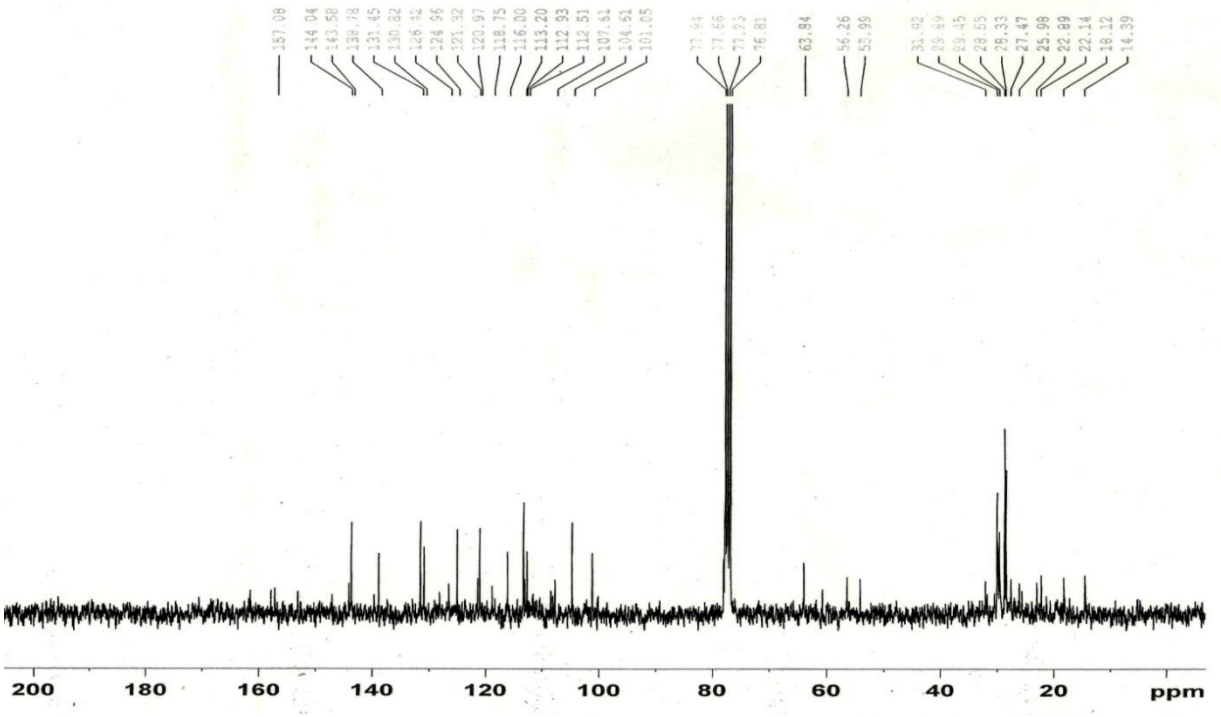


Fig. 23: ^{13}C NMR Spectral data of Osthol

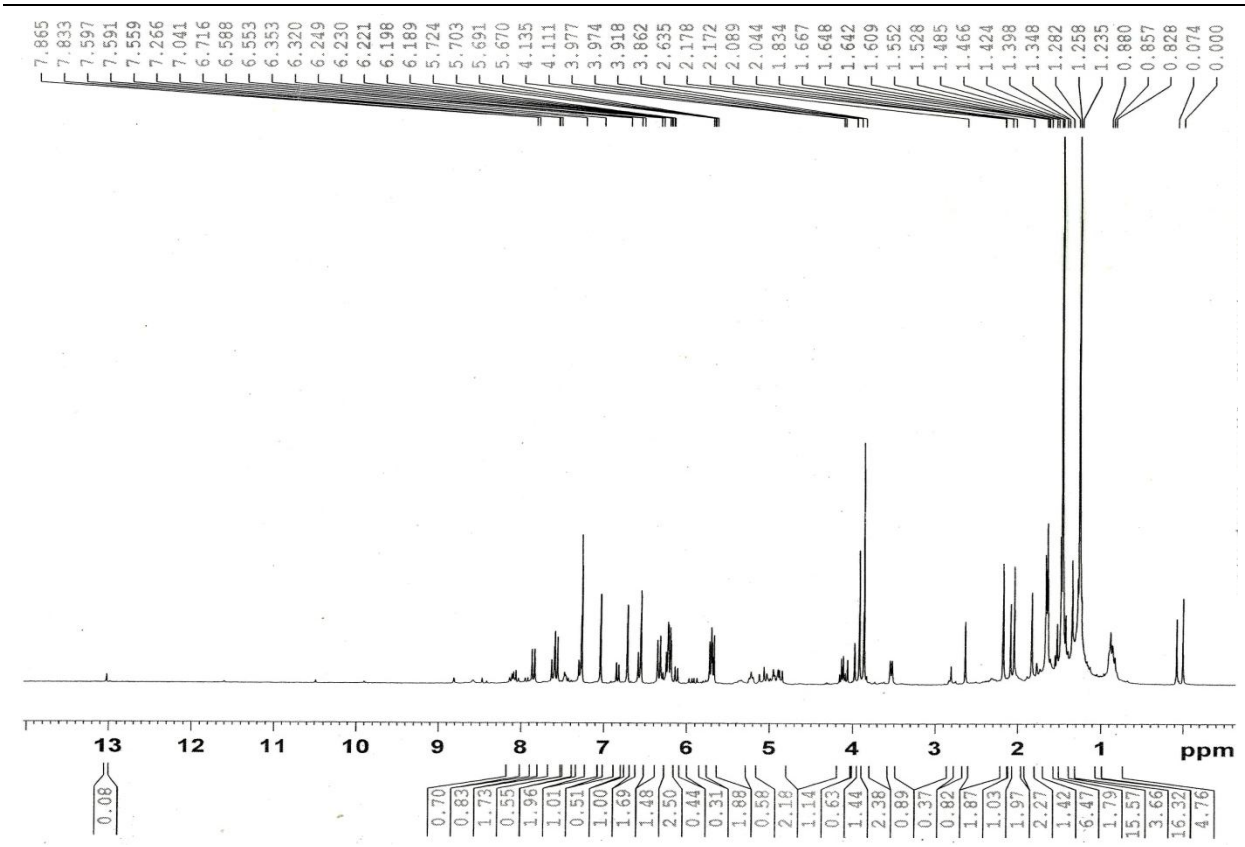


Fig. 24: ^{1}H NMR Spectral data of Osthol

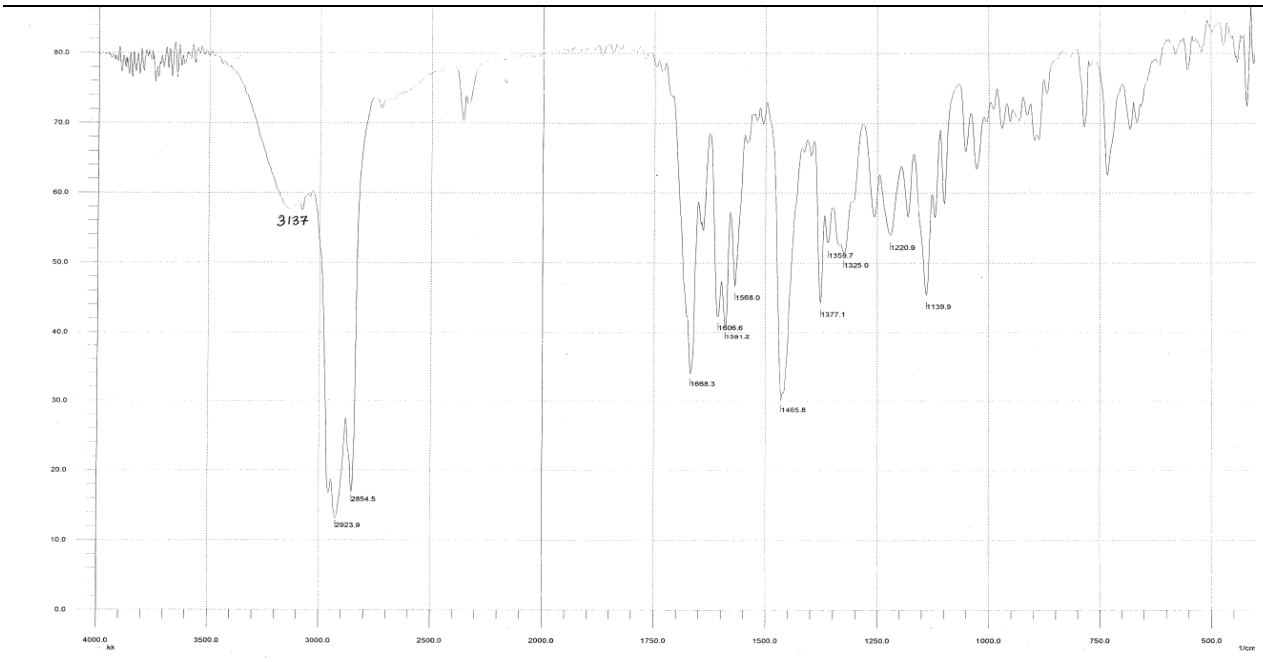


Fig. 25: IR specrtal data of Clausarin

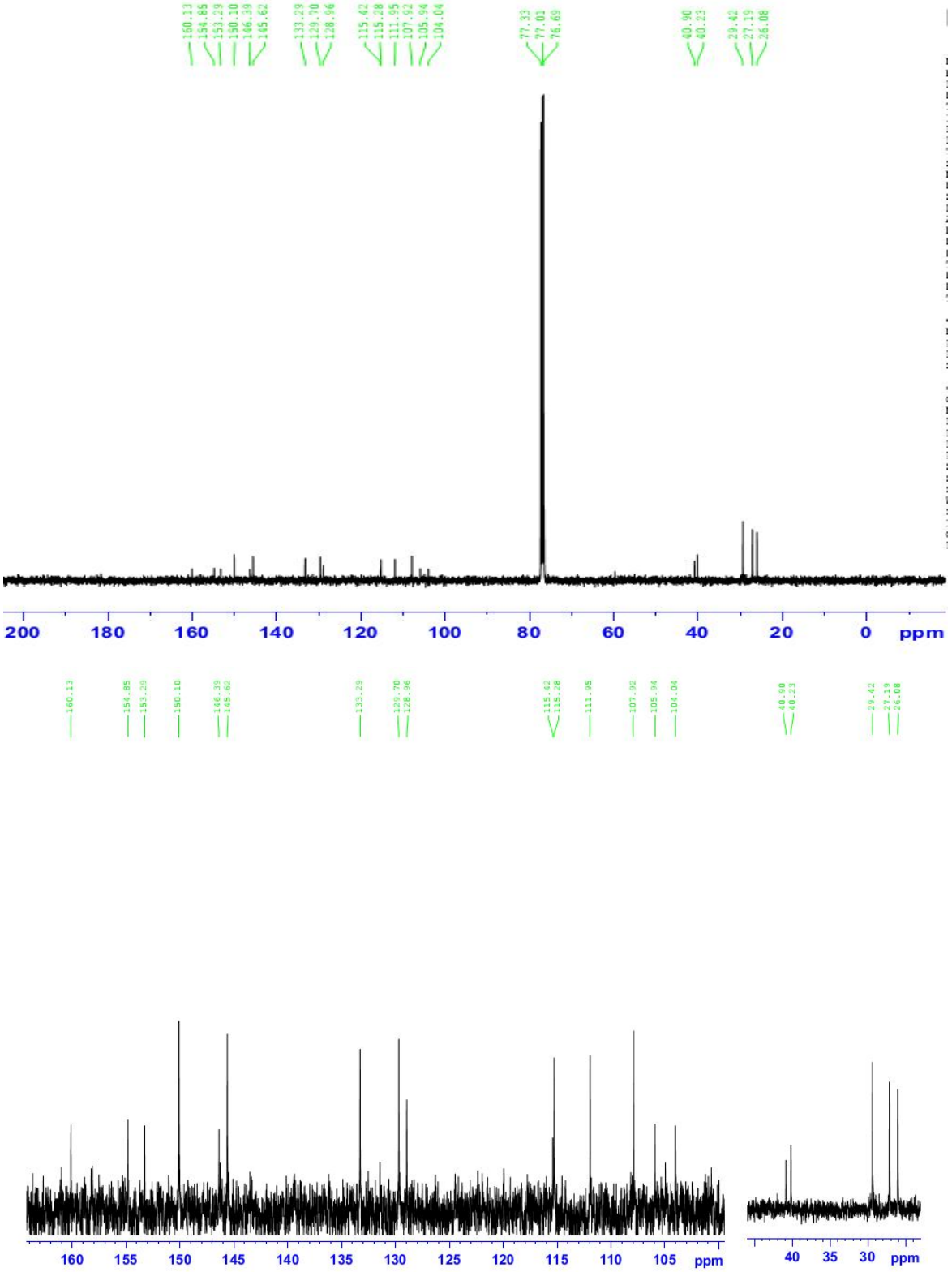


Fig. 26: ^{13}C NMR Spectral data of Clausarin

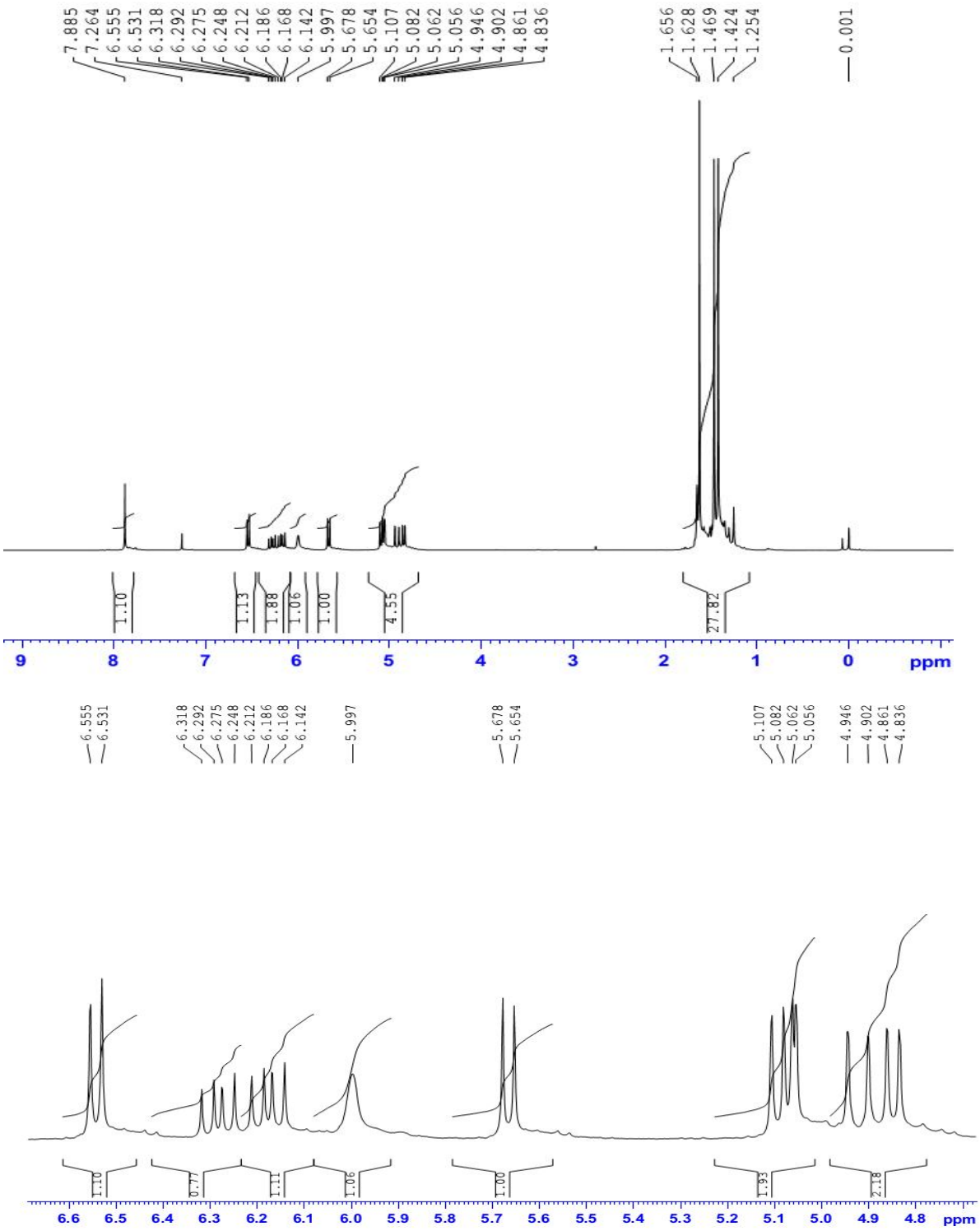


Fig. 27: ^{1}H NMR Spectral data of Clausarin

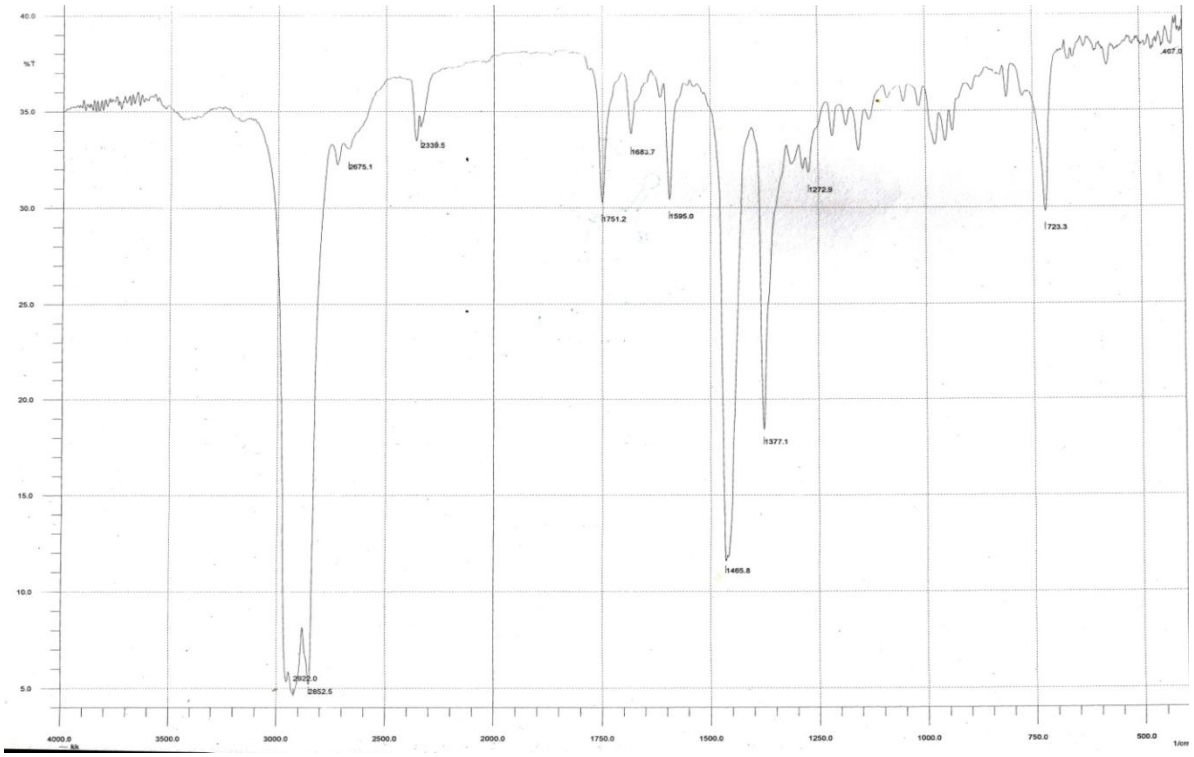


Fig. 28: IR spectral data of 8-*epi*-xanthatin

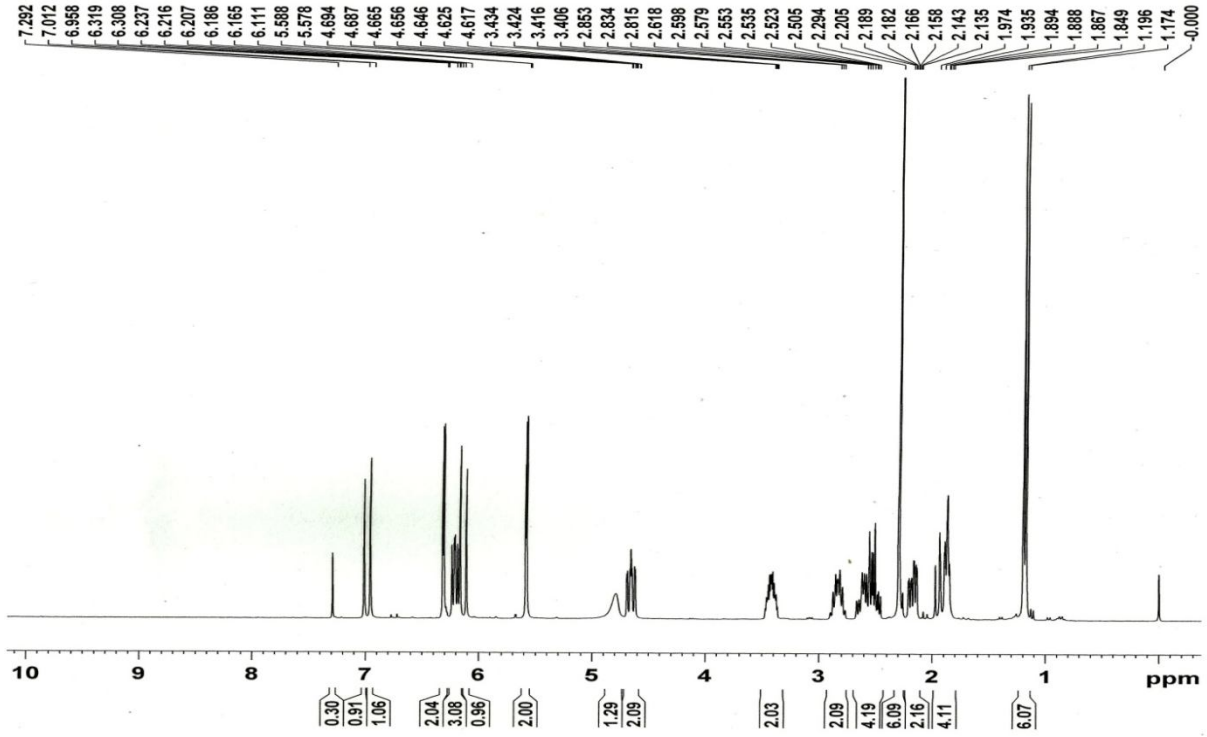


Fig. 29: ^{1}H NMR spectral data of 8-*epi*-xanthatin

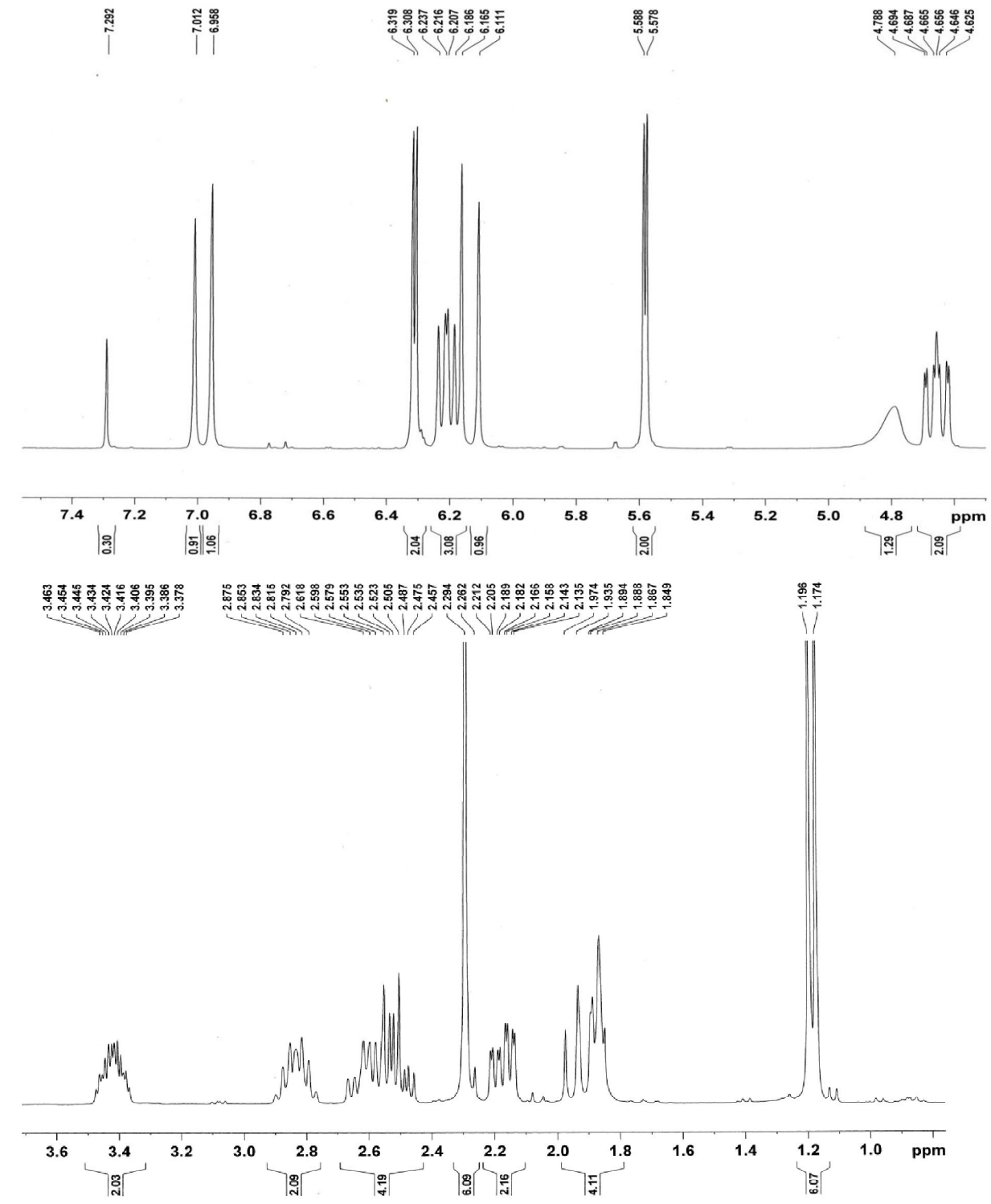


Fig. 30: ^{1}H NMR spectral data of 8-*epi*-xanthatin

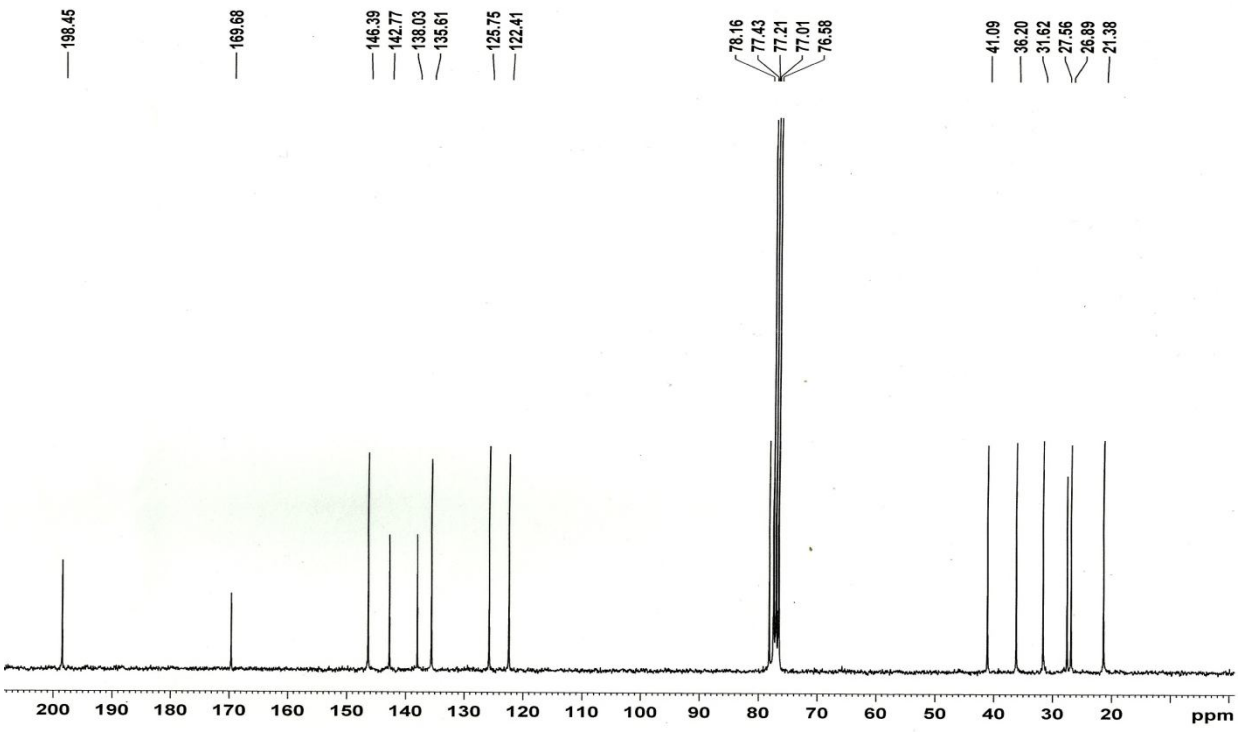


Fig. 31: ^{13}C NMR spectral data of 8-*epi*-xanthatin

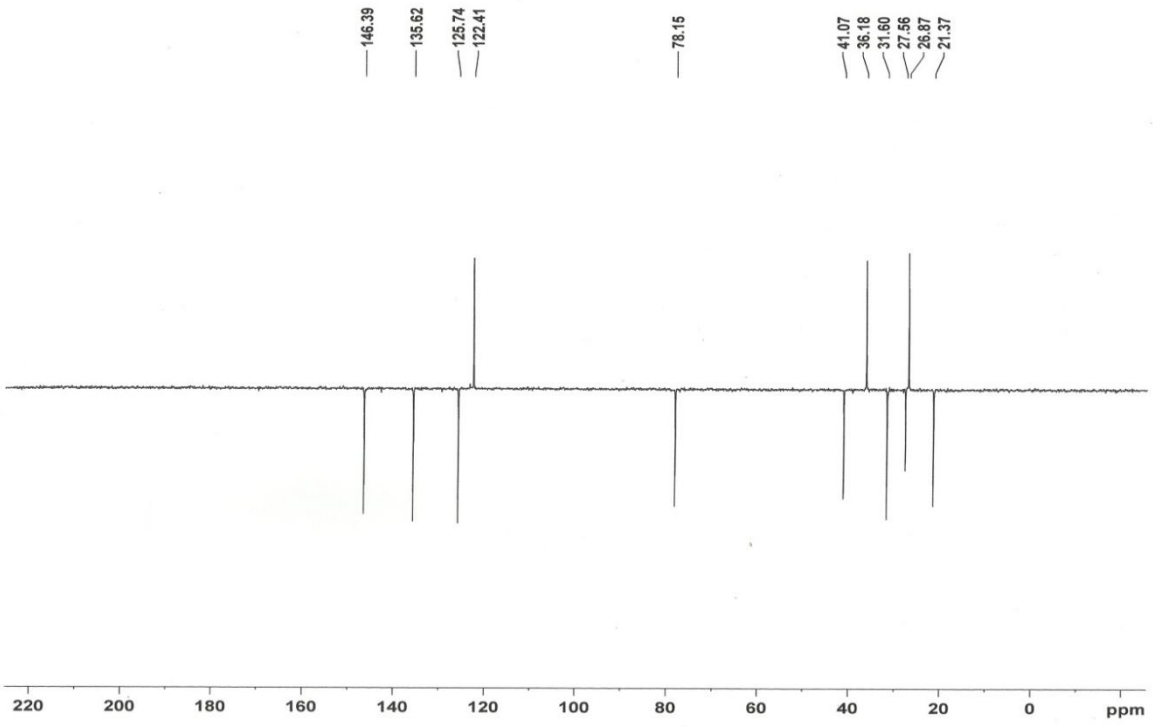


Fig. 32: DEPT-135 spectral data 8-*epi*-xanthatin

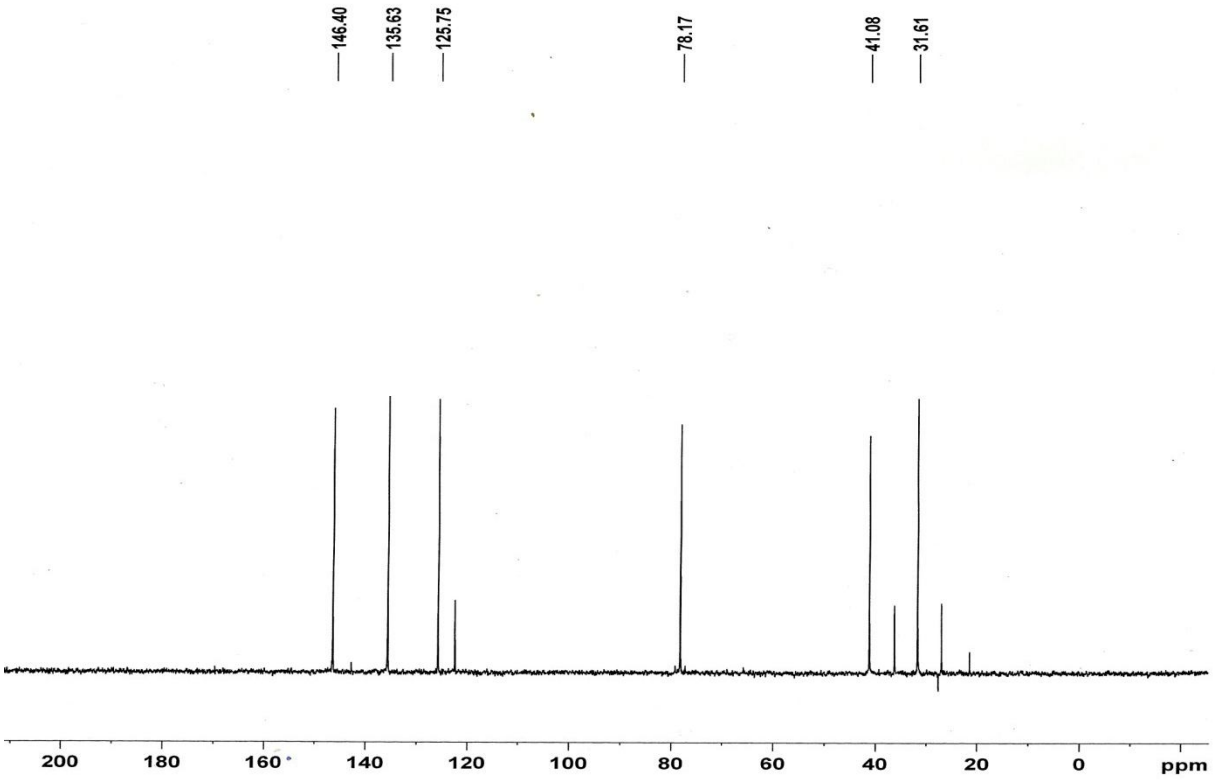


Fig. 33: DEPT-90 spectral data of 8-*epi*-xanthatin

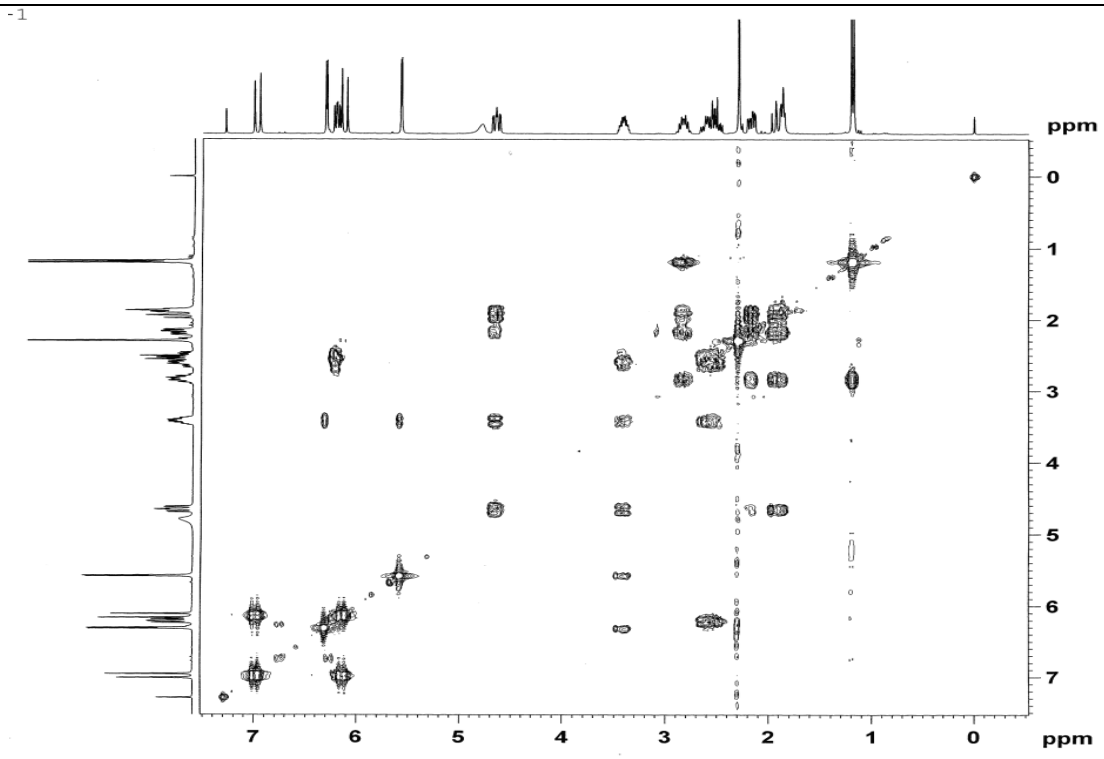


Fig. 34: COSY spectral data of 8-*epi*-xanthatin

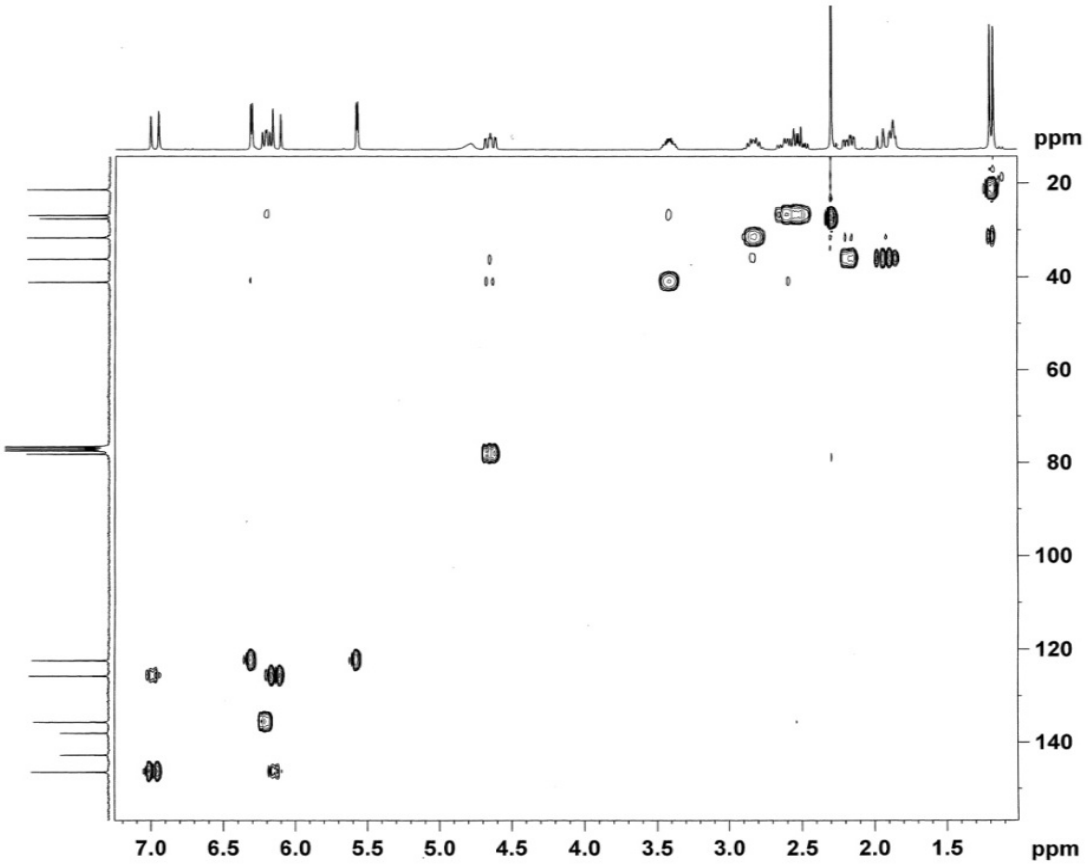


Fig. 35: HETCOR spectral data of 8-*epi*-xanthatin

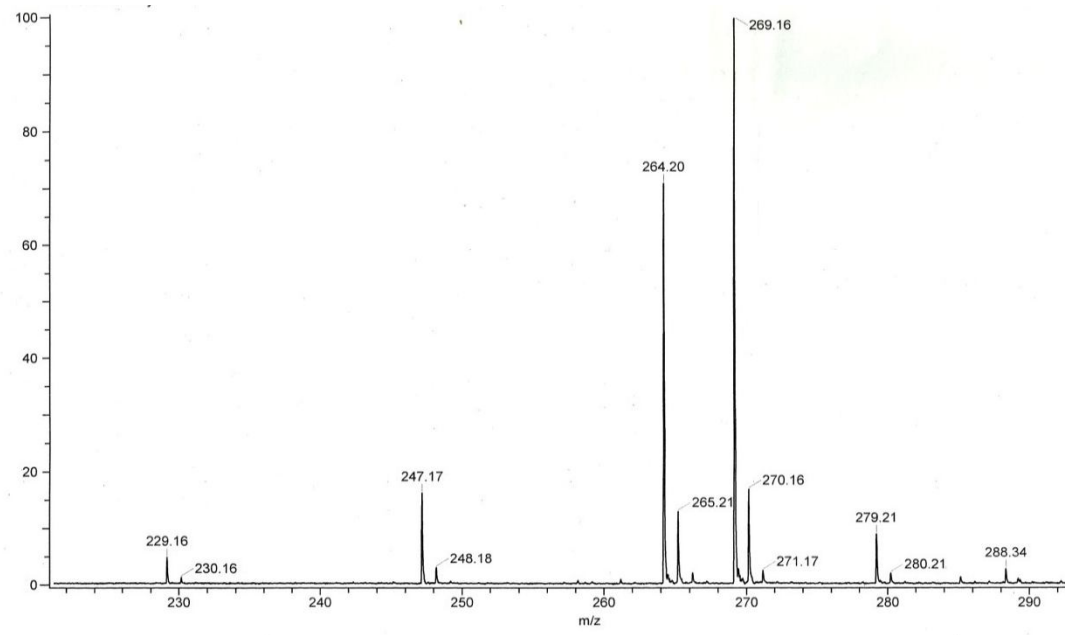


Fig. 36: ESI-MS spectral data of 8-*epi*-xanthatin

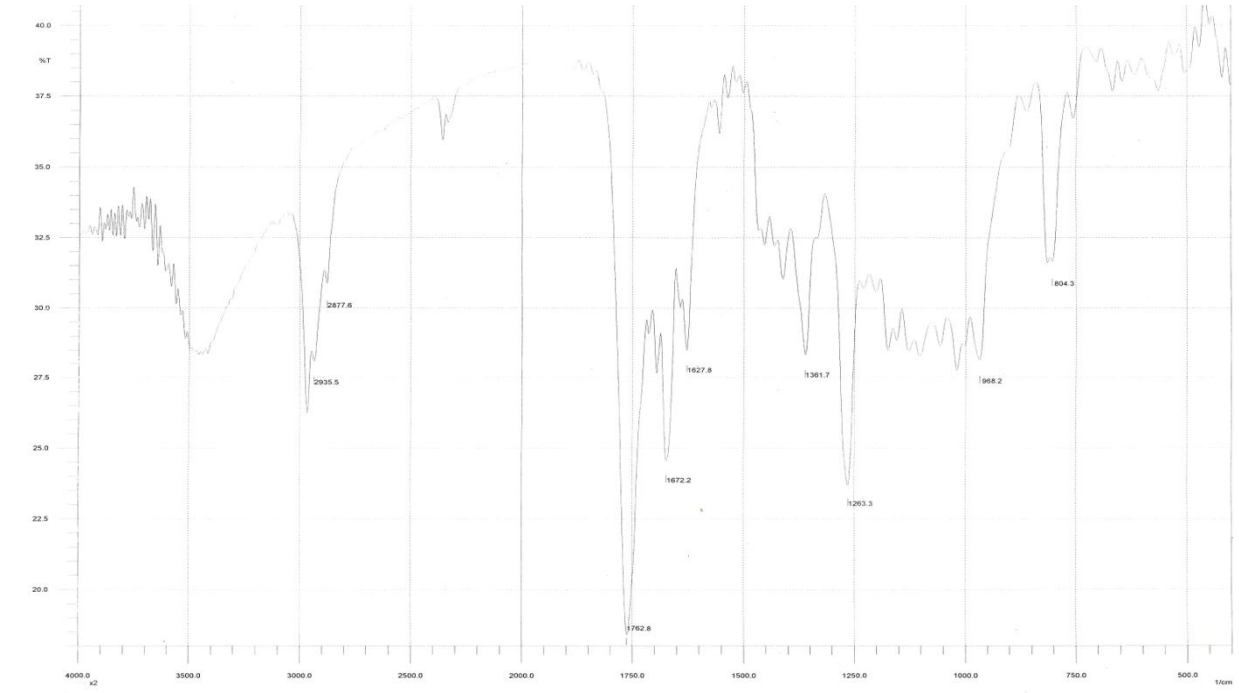


Fig. 37: IR spectral data of 8-*epi*-xanthatin-1ß,5ß-epoxide

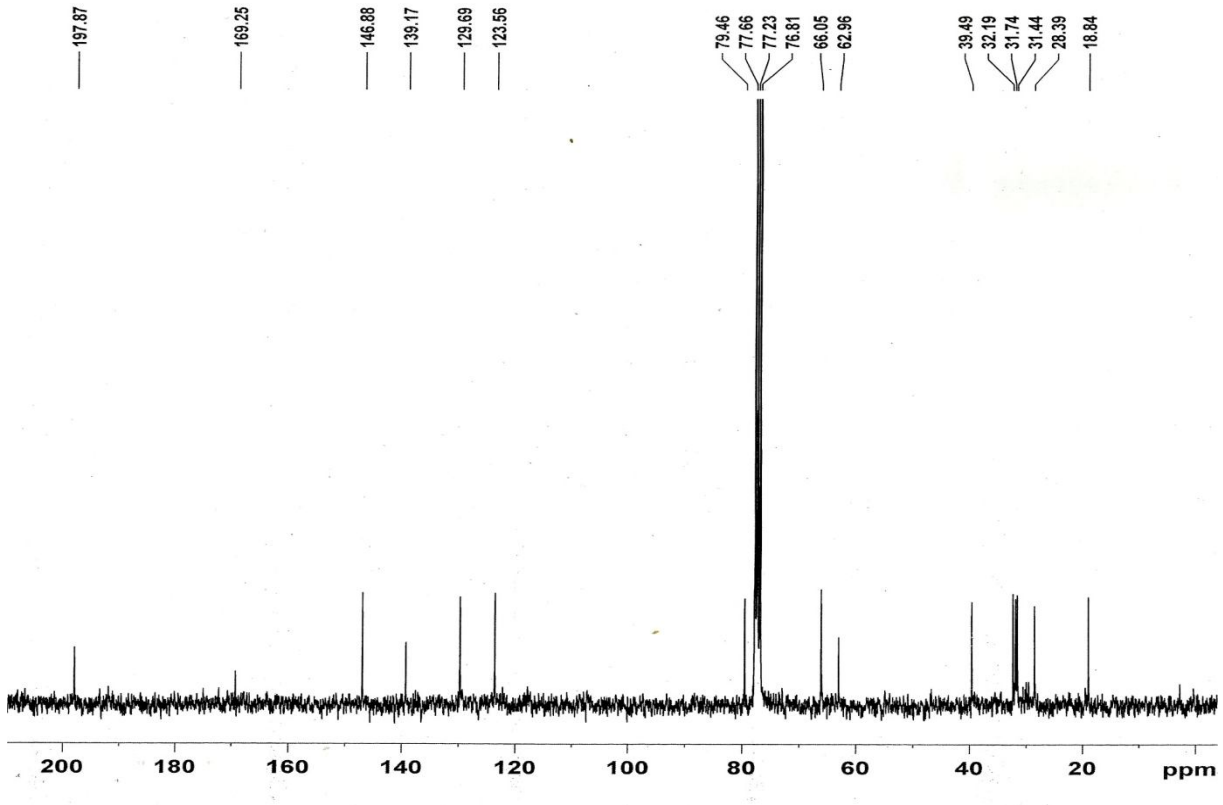


Fig. 38: ^{13}C NMR spectral data of 8-*epi*-xanthatin-1ß,5ß-epoxide

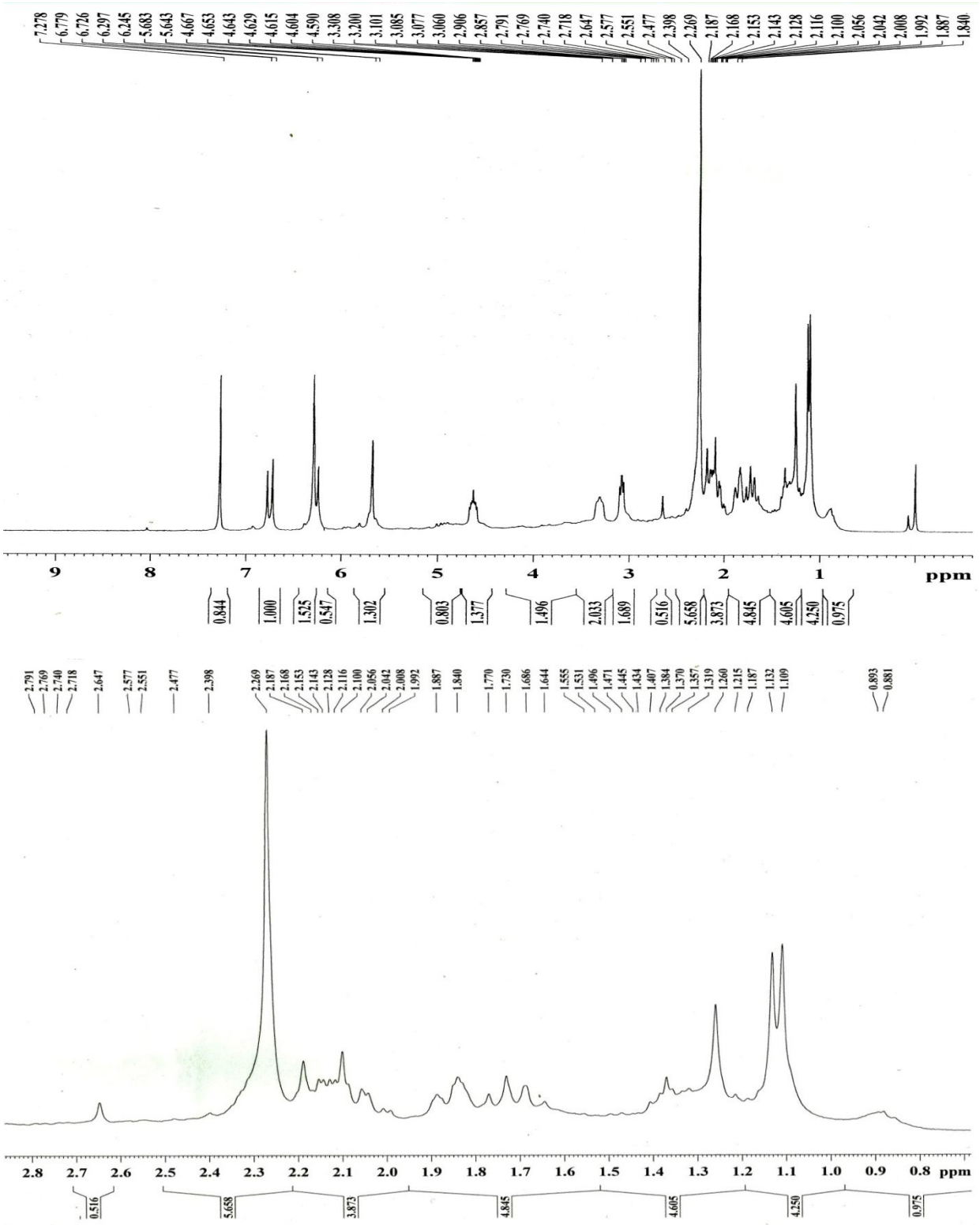


Fig. 39: ^{1}H NMR spectral data of 8-*epi*-xanthatin-1ß,5ß-epoxide

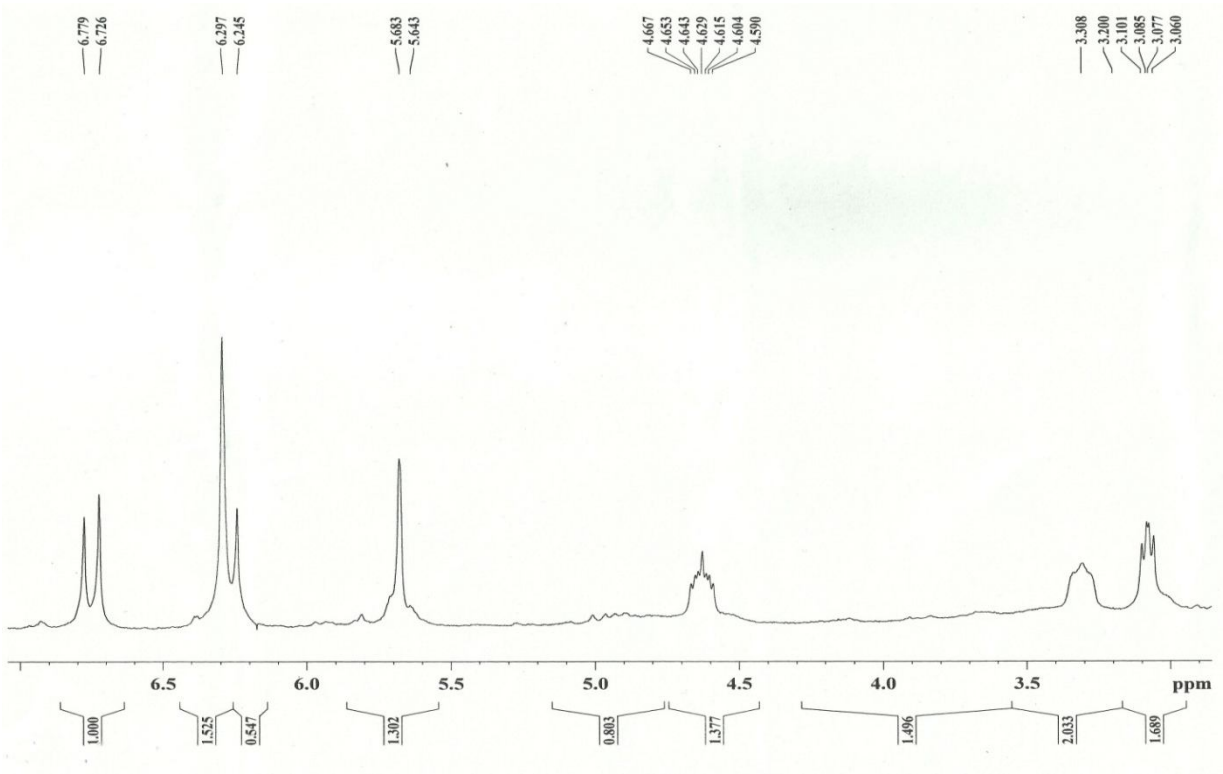


Fig. 40: ^{1}H NMR spectral data of 8-*epi*-xanthatin-1ß,5ß-epoxide

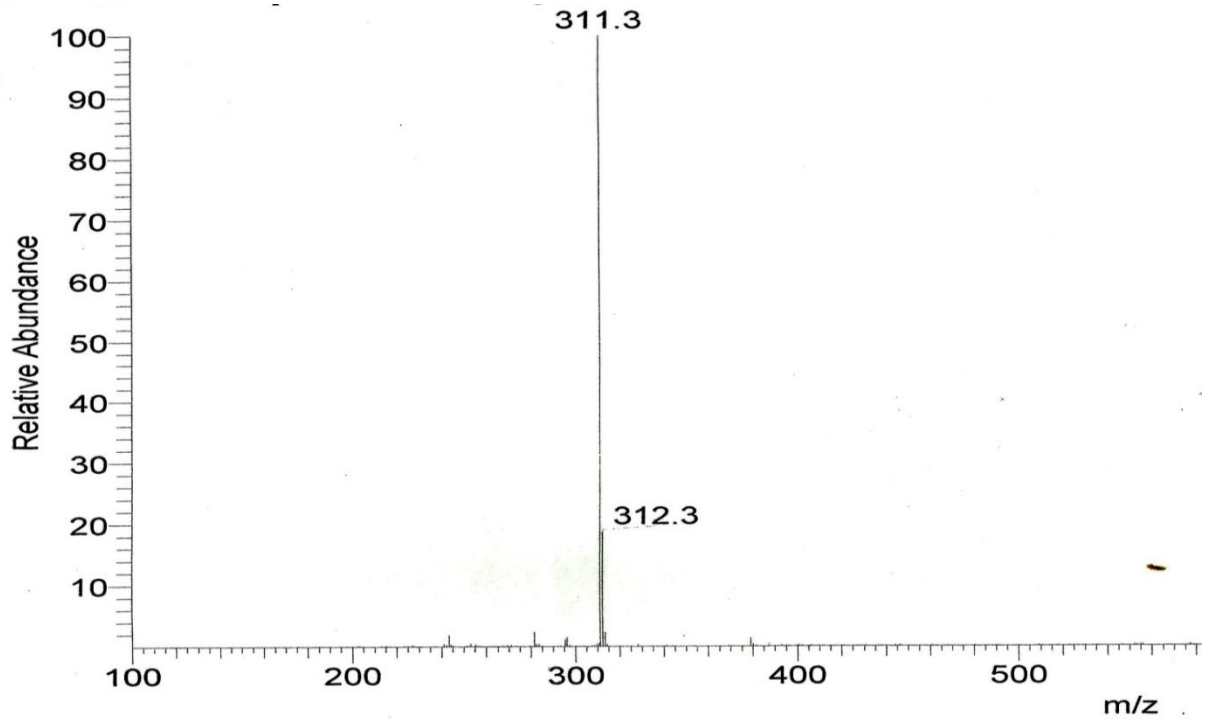


Fig. 41: ESI-MS spectral data of 8-*epi*-xanthatin-1ß,5ß-epoxide

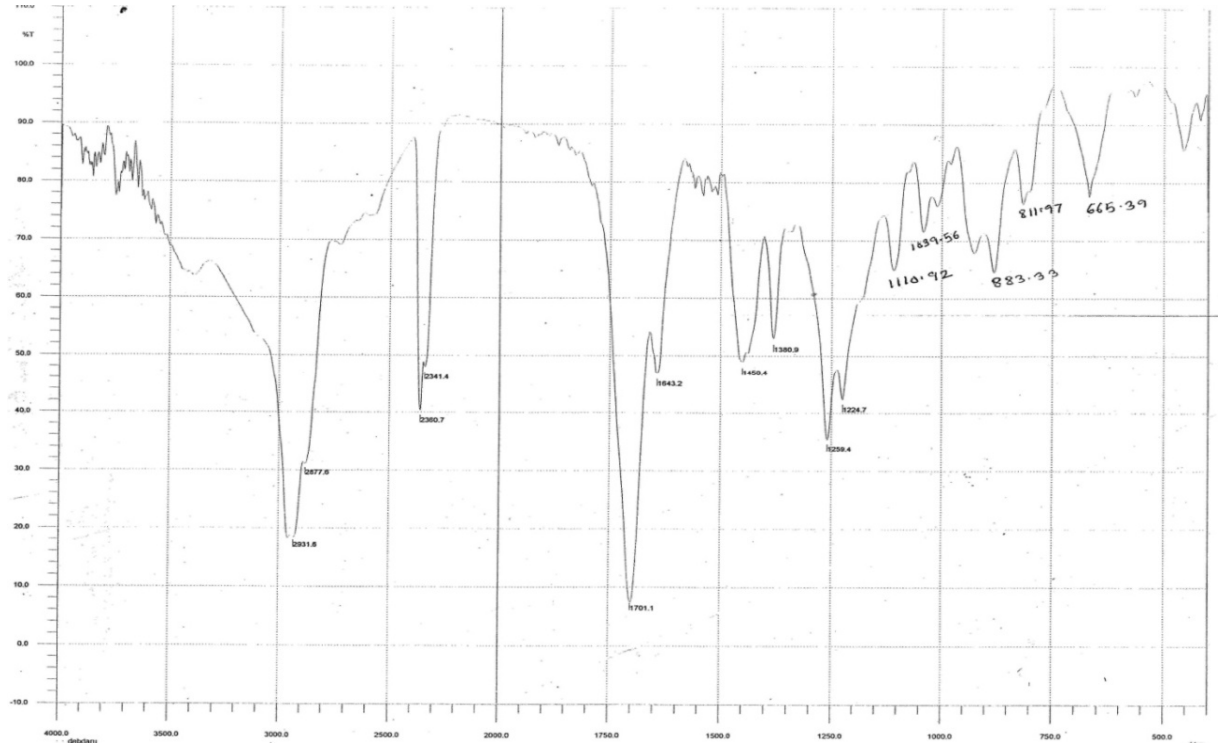


Fig. 42: IR spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

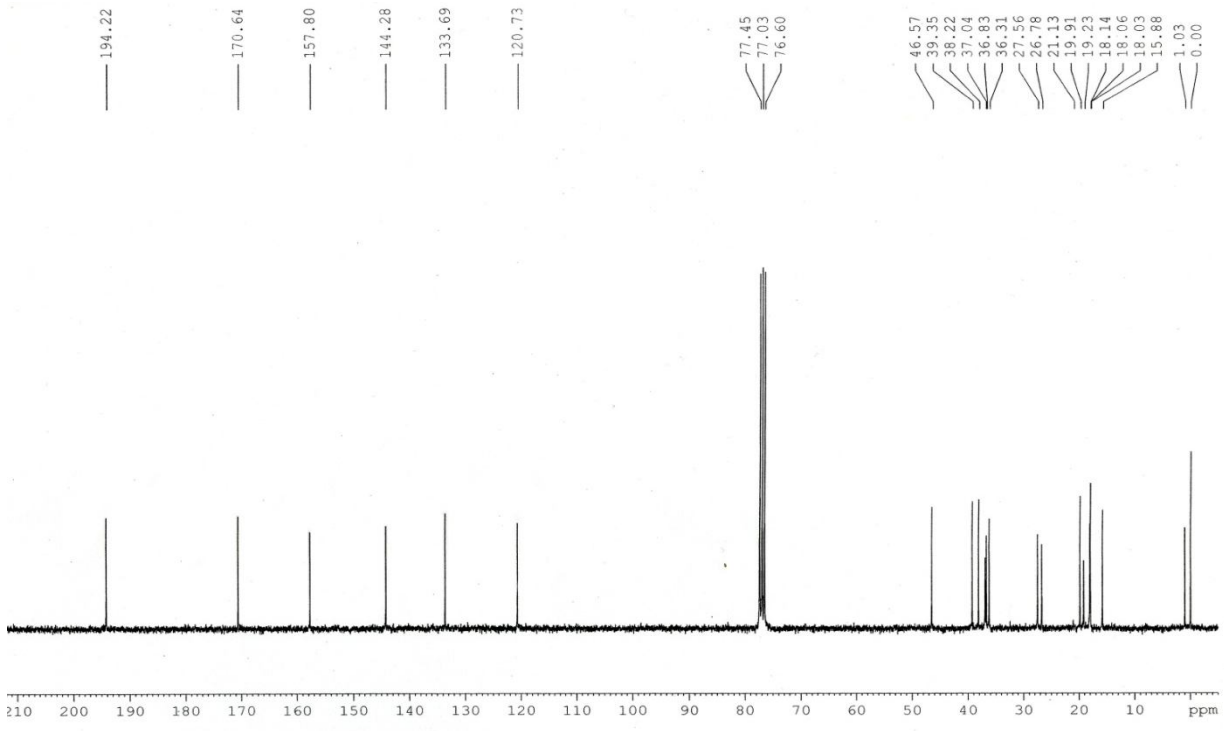


Fig. 43: ^{13} C NMR spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

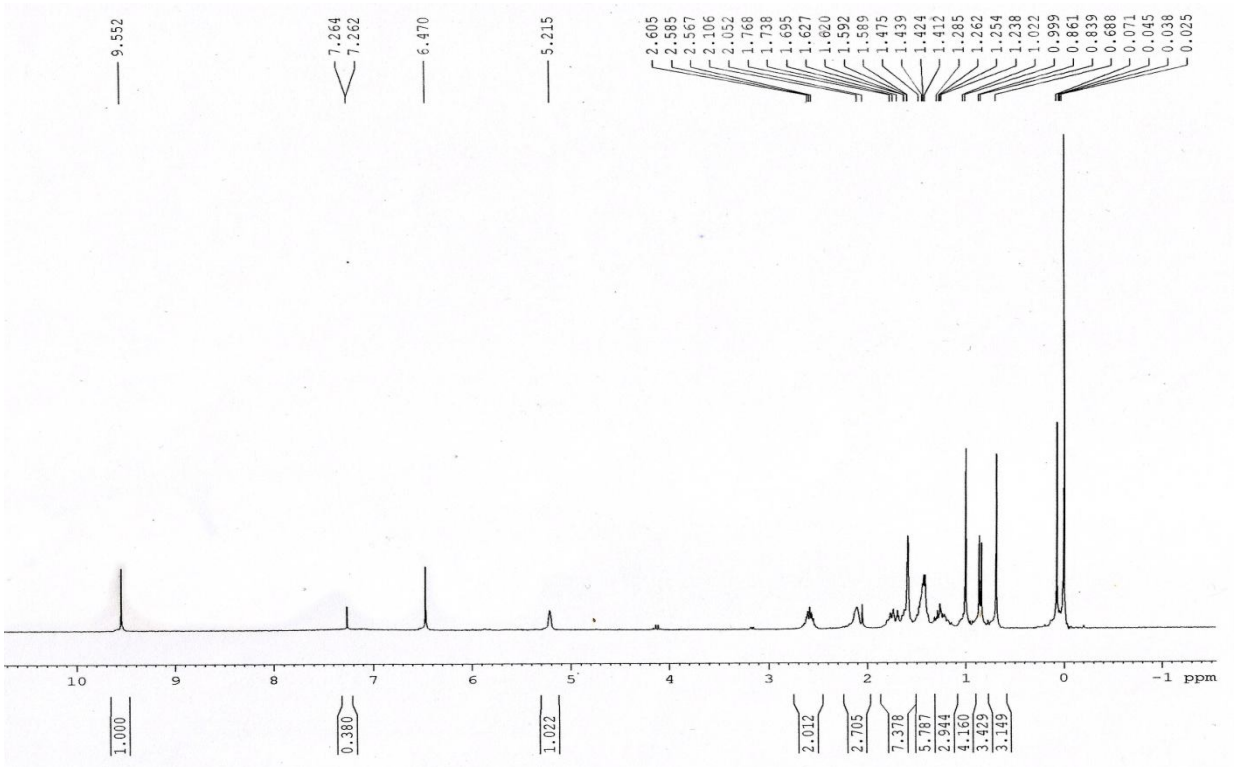


Fig. 44: ^{1}H NMR spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

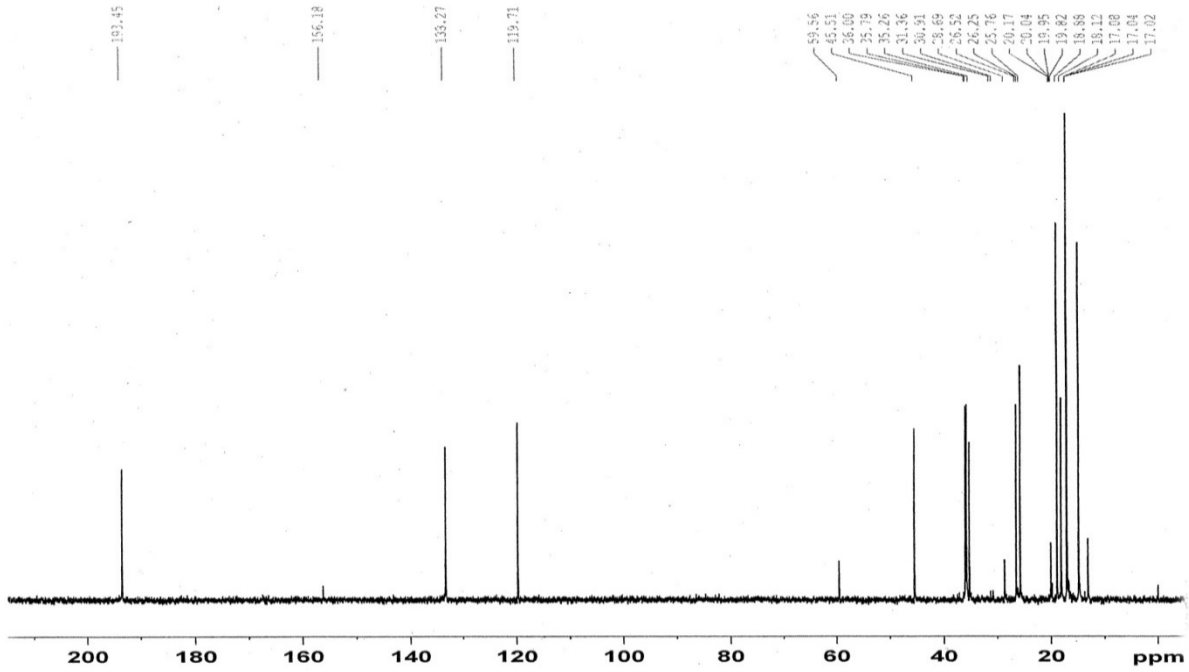


Fig. 45: DEPT-45 spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

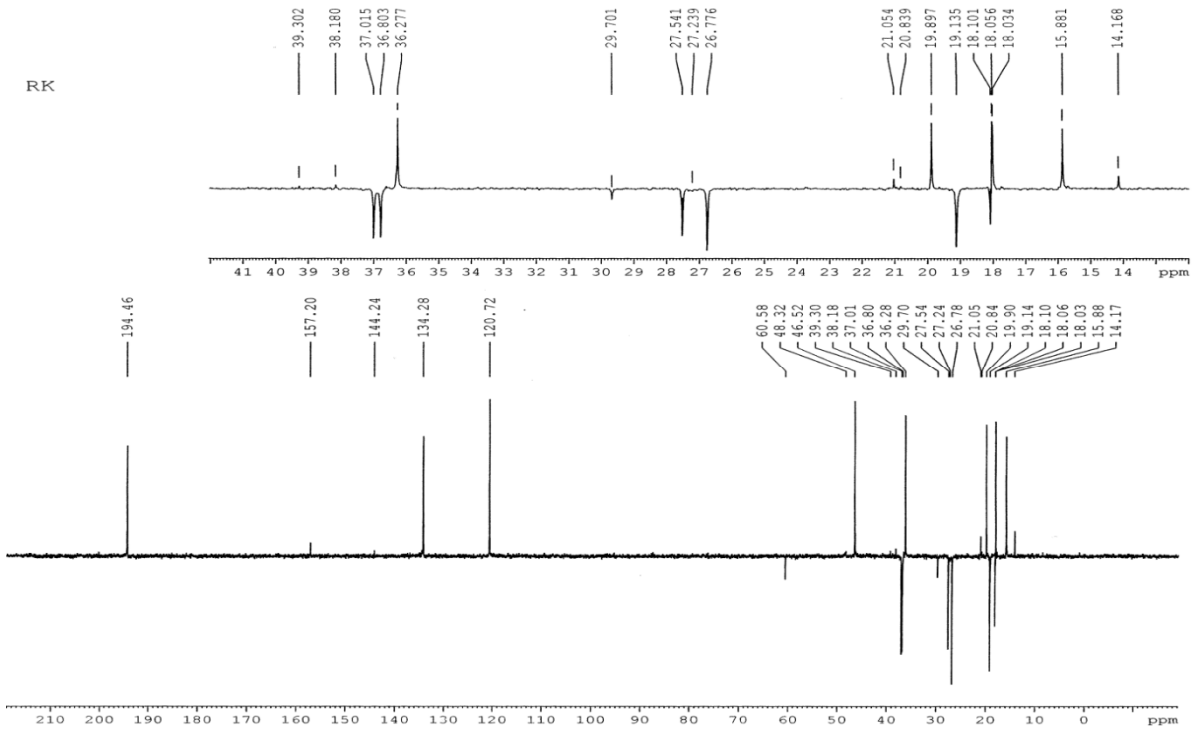


Fig. 46: DEPT-135 spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

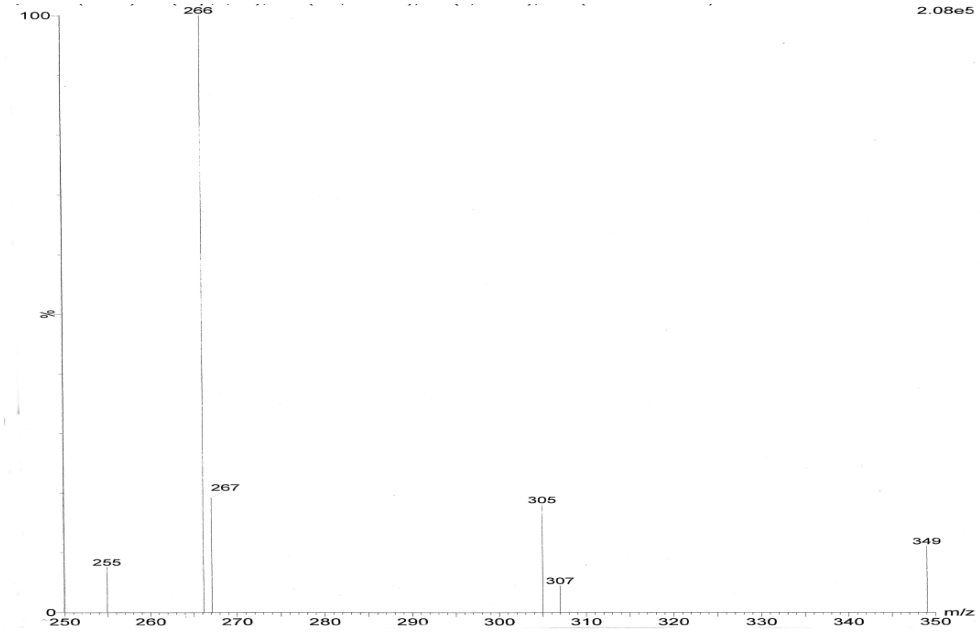


Fig. 47: ESI-MS spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

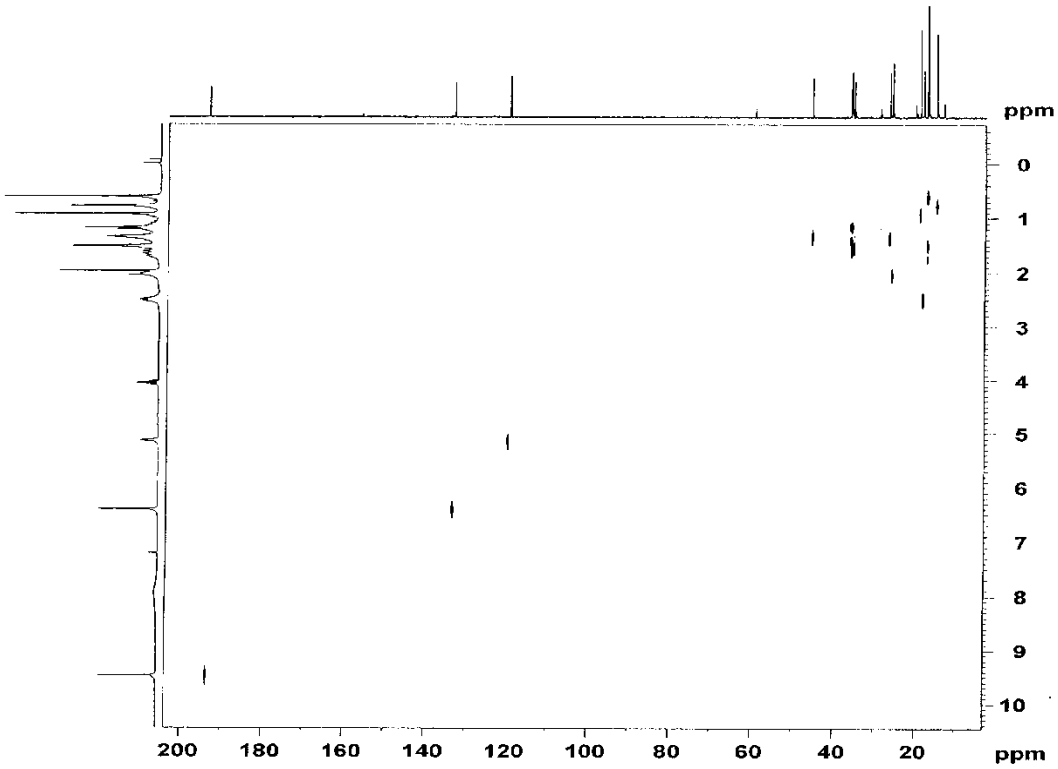


Fig. 48: COSY spectral data of 16-Oxocleroda-3,13(14)E-dien-15-oic acid

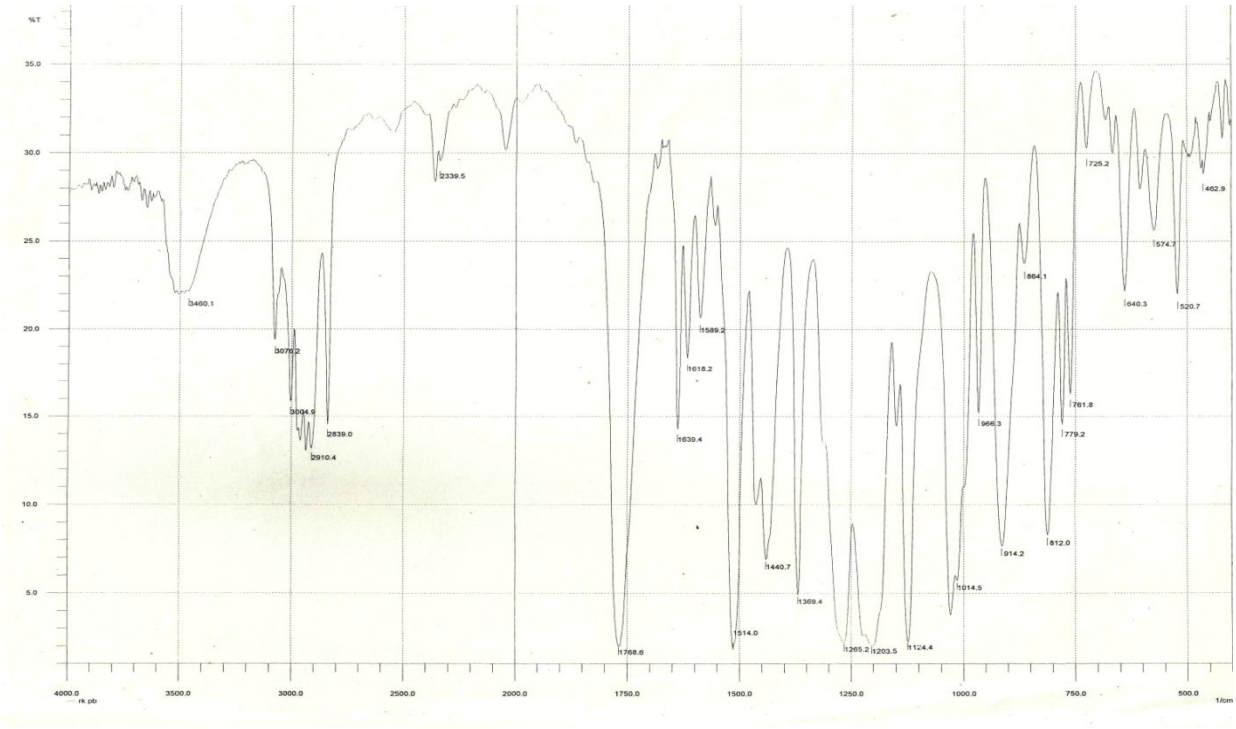


Fig. 49: IR spectral data of Chavibetol acetate

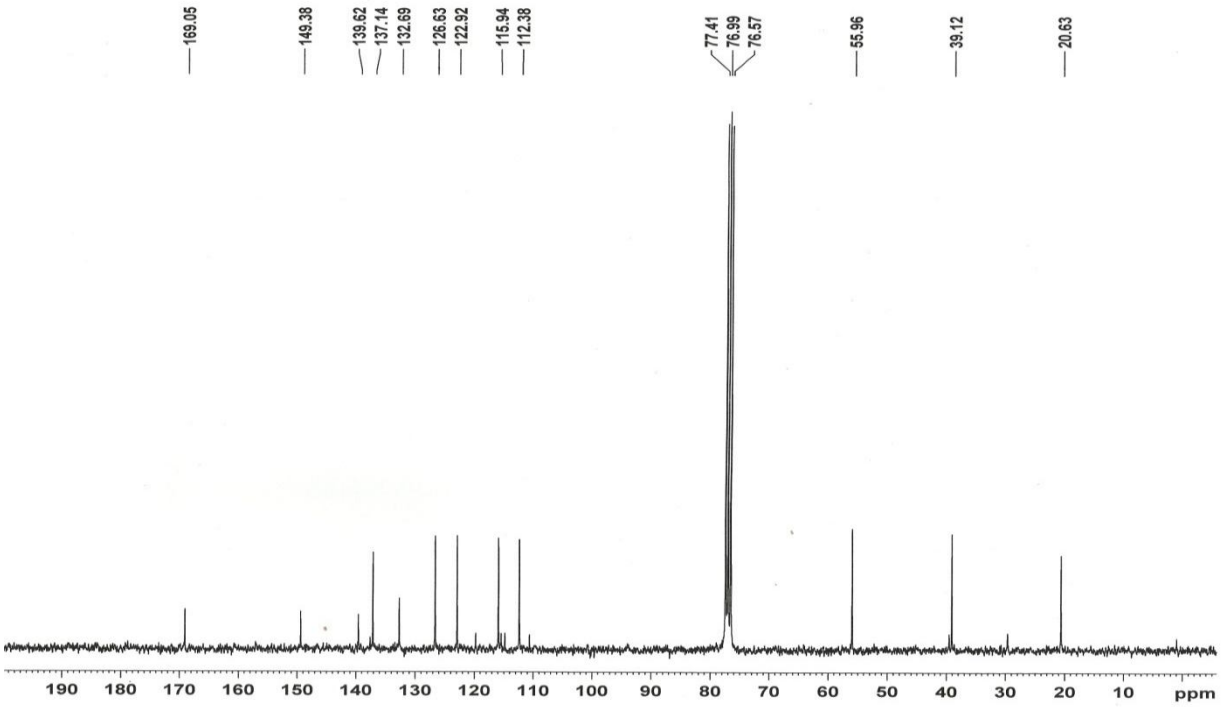


Fig. 50: ^{13}C NMR spectral data of Chavibetol acetate

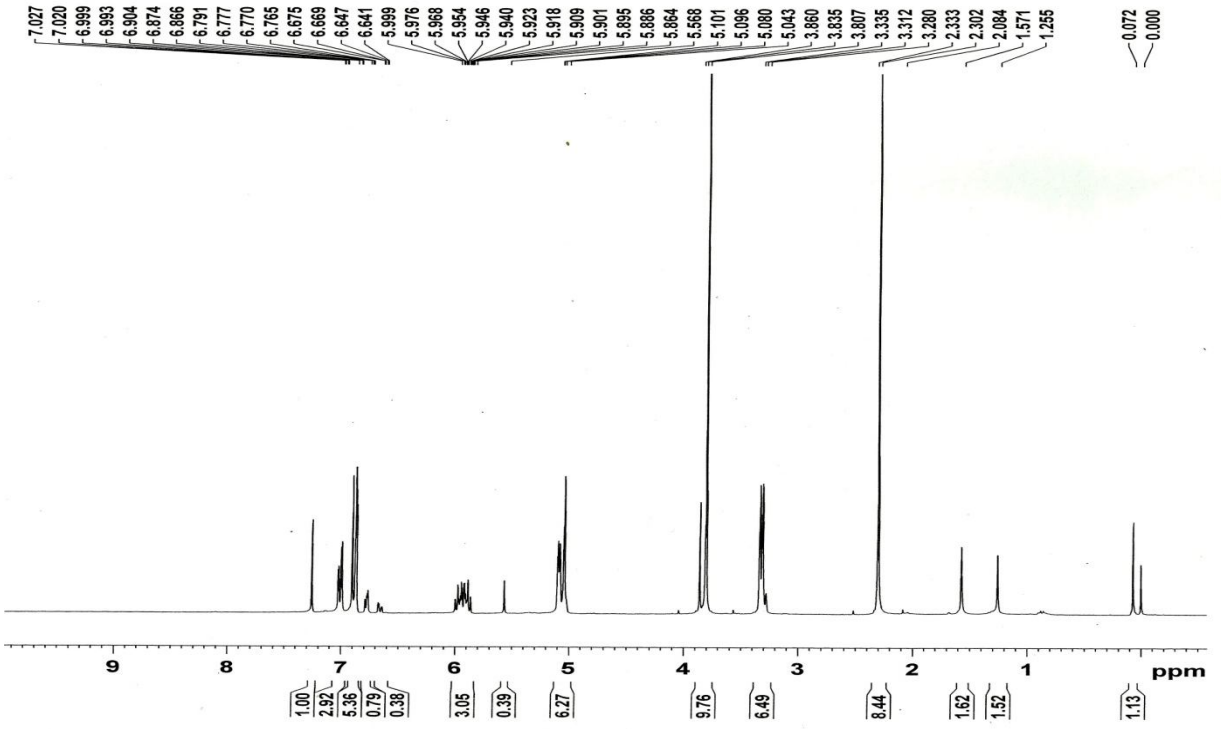


Fig. 51: ^{1}H NMR spectral data of Chavibetol acetate

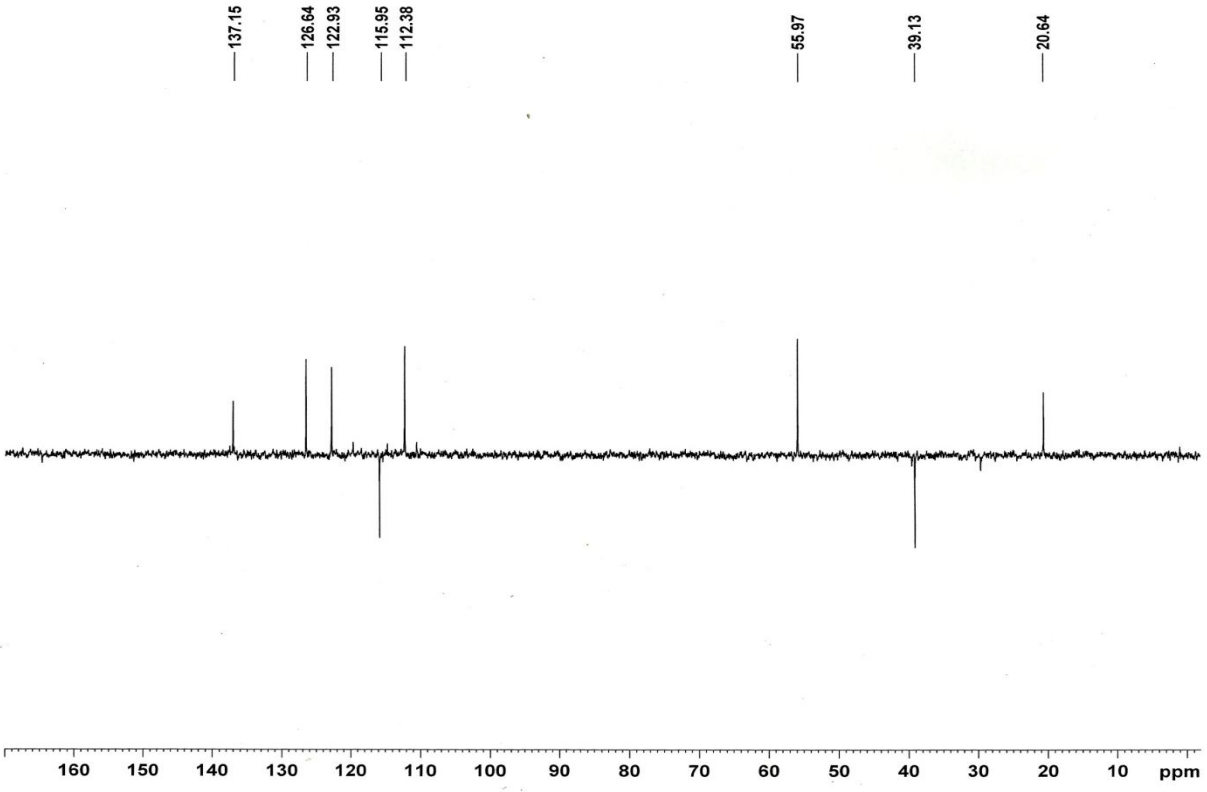


Fig. 52: DEPT-135 spectral data of Chavibetol acetate

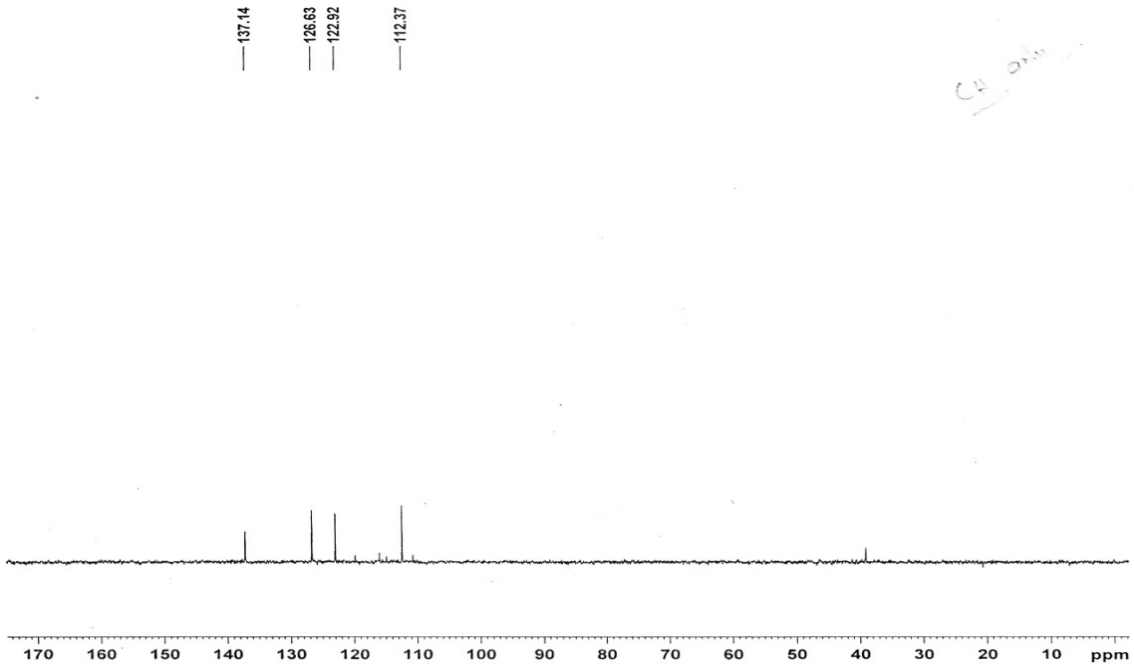


Fig. 53: DEPT-90 spectra of Chavibetol acetate

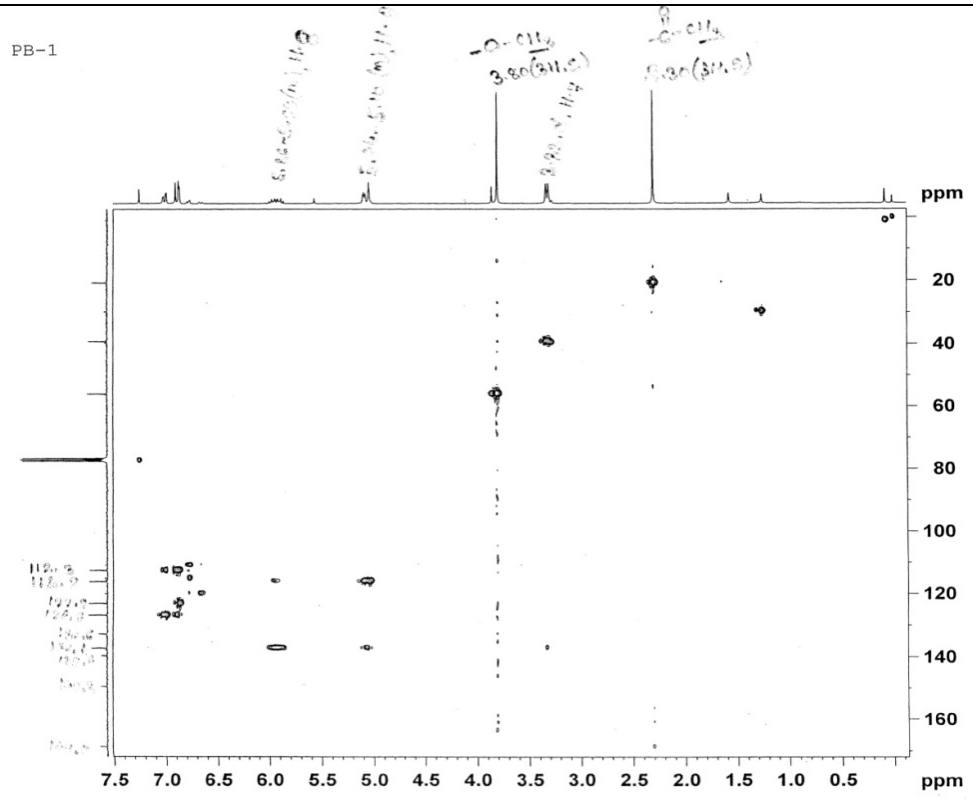


Fig. 54: COSY spectral data of Chavibetol acetate

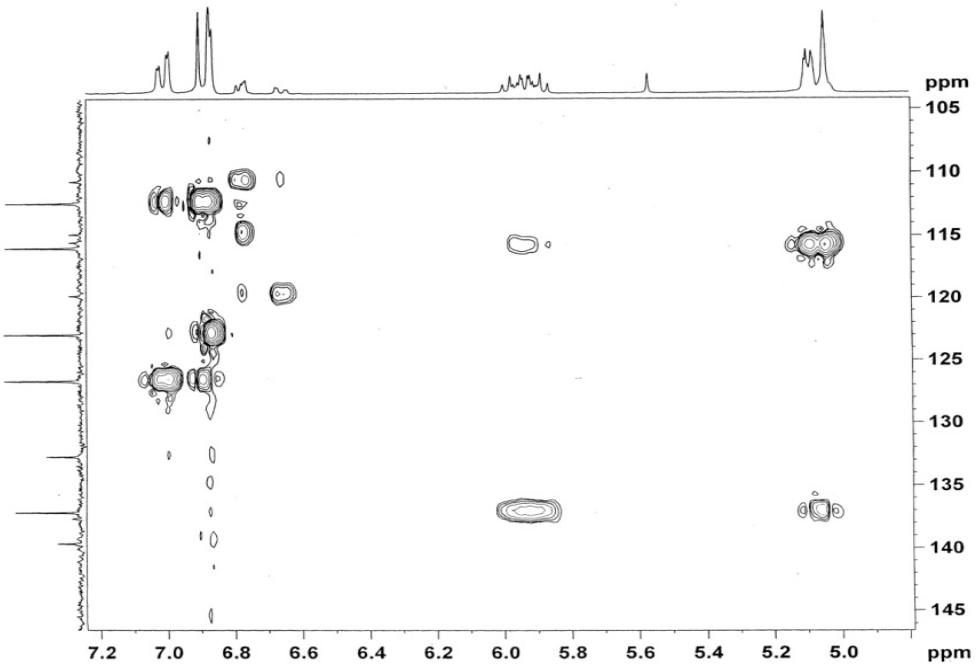


Fig. 55: HETCOR spectral data of Chavibetol acetate

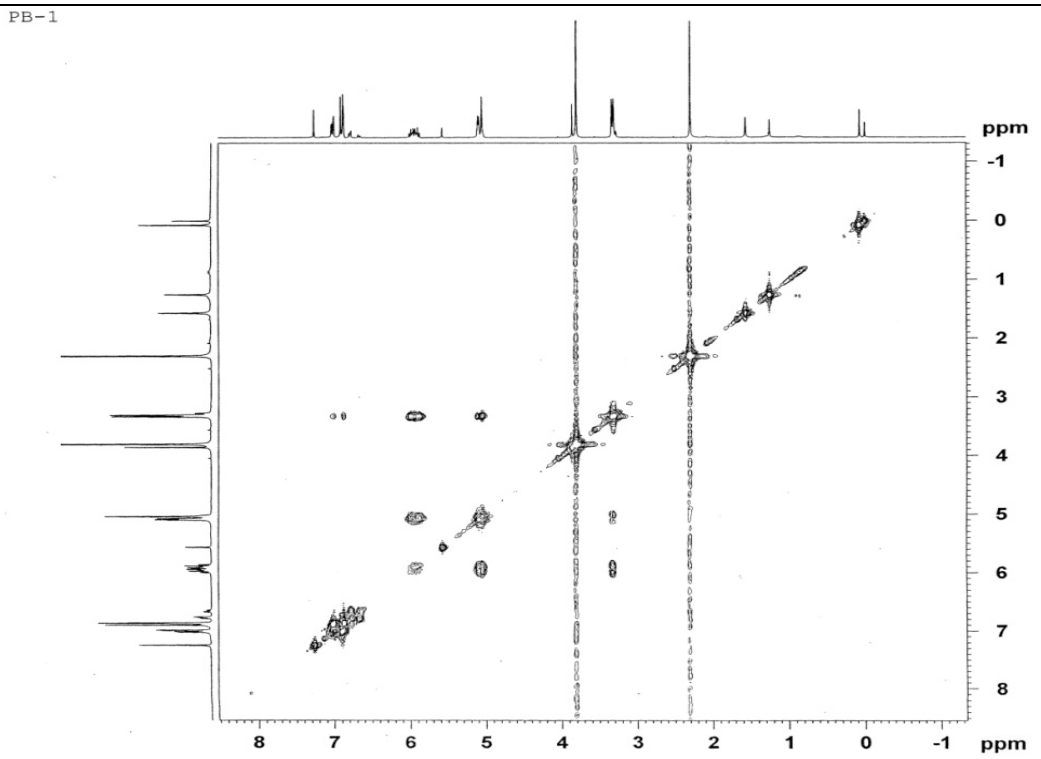


Fig. 56: ^{1}H COSY spectral data of Chavibetol acetate

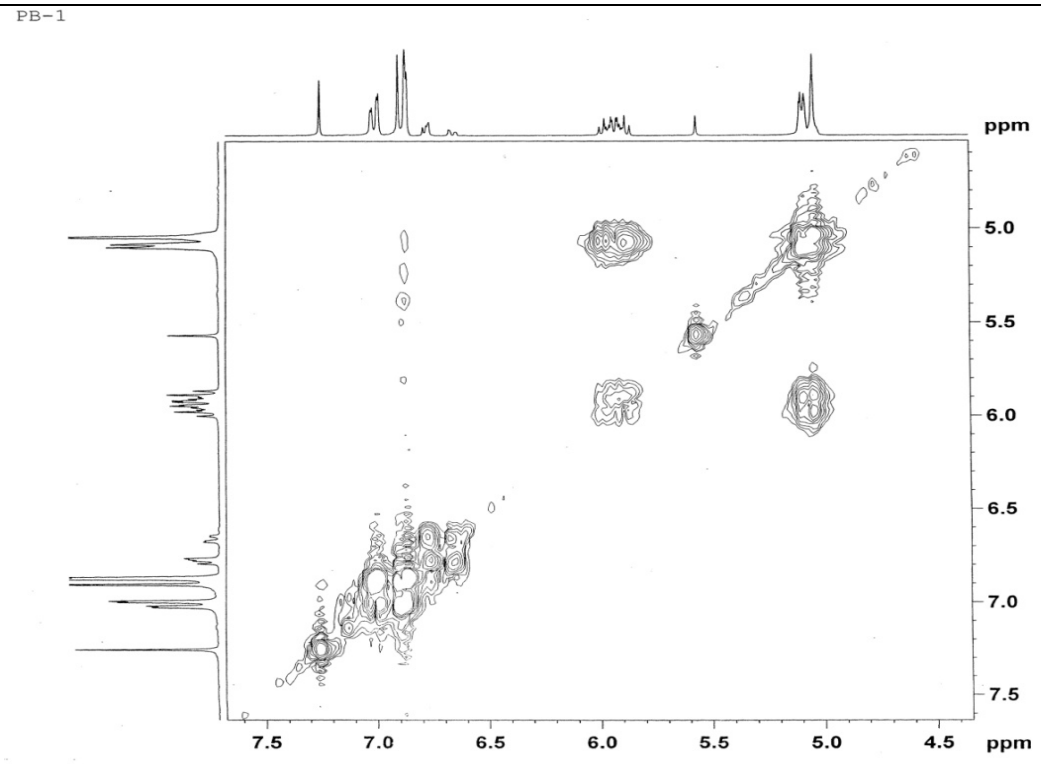


Fig. 57: ^{1}H COSY spectral data of Chavibetol acetate

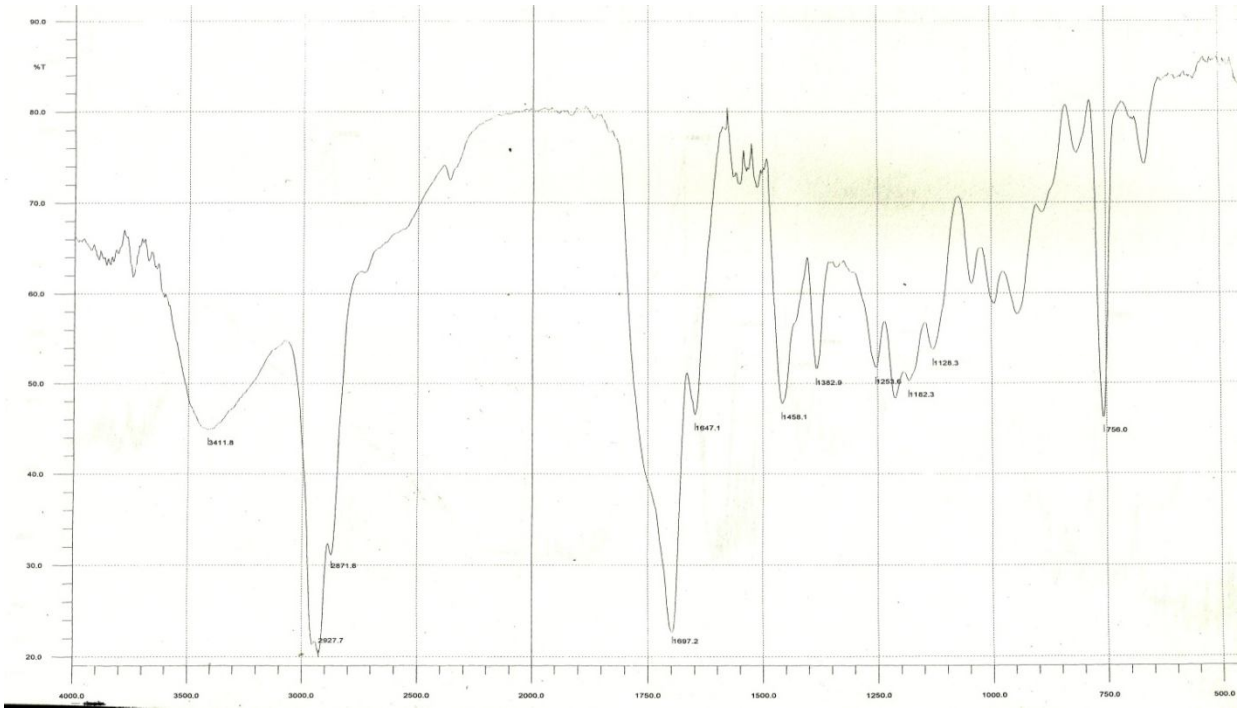


Fig. 58: IR spectral data of 18-Hydroxy-16-oxocleroda-3, 13(14) E-dien-15-oic acid (Biotransformed products)

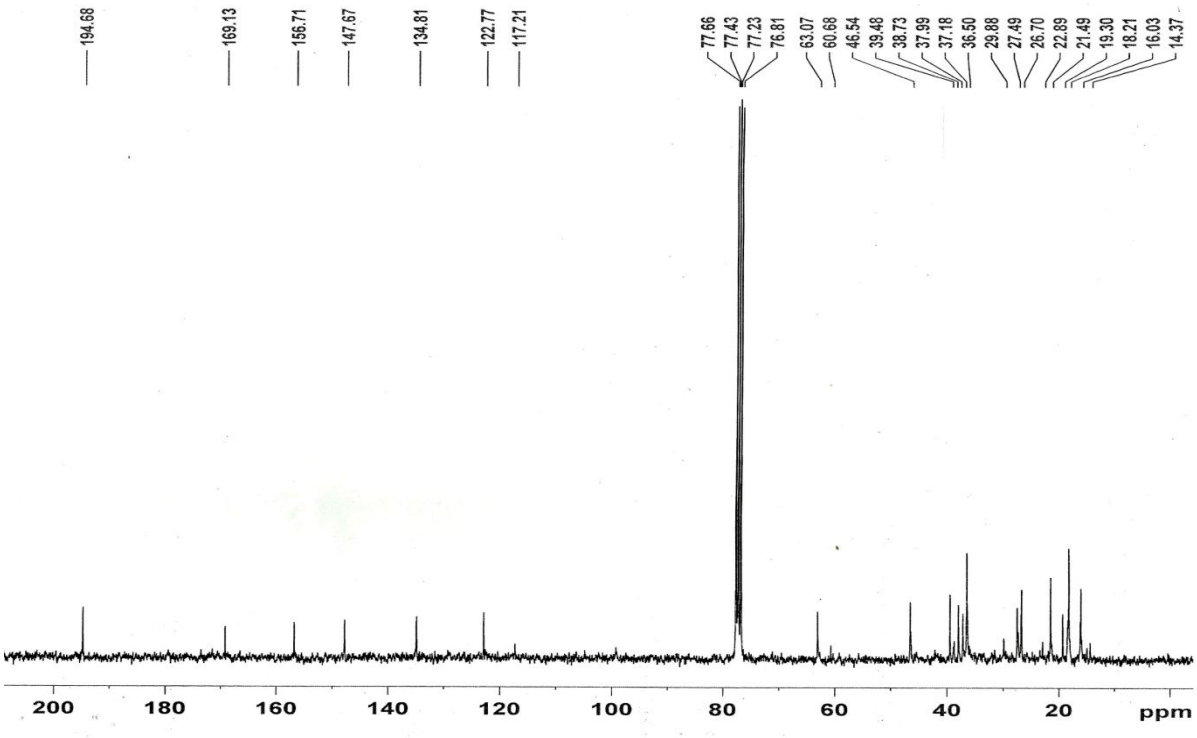


Fig. 59: ^{13}C NMR spectral data of 18-Hydroxy-16-oxocleroda-3, 13(14) E-dien-15-oic acid (Biotransformed products)

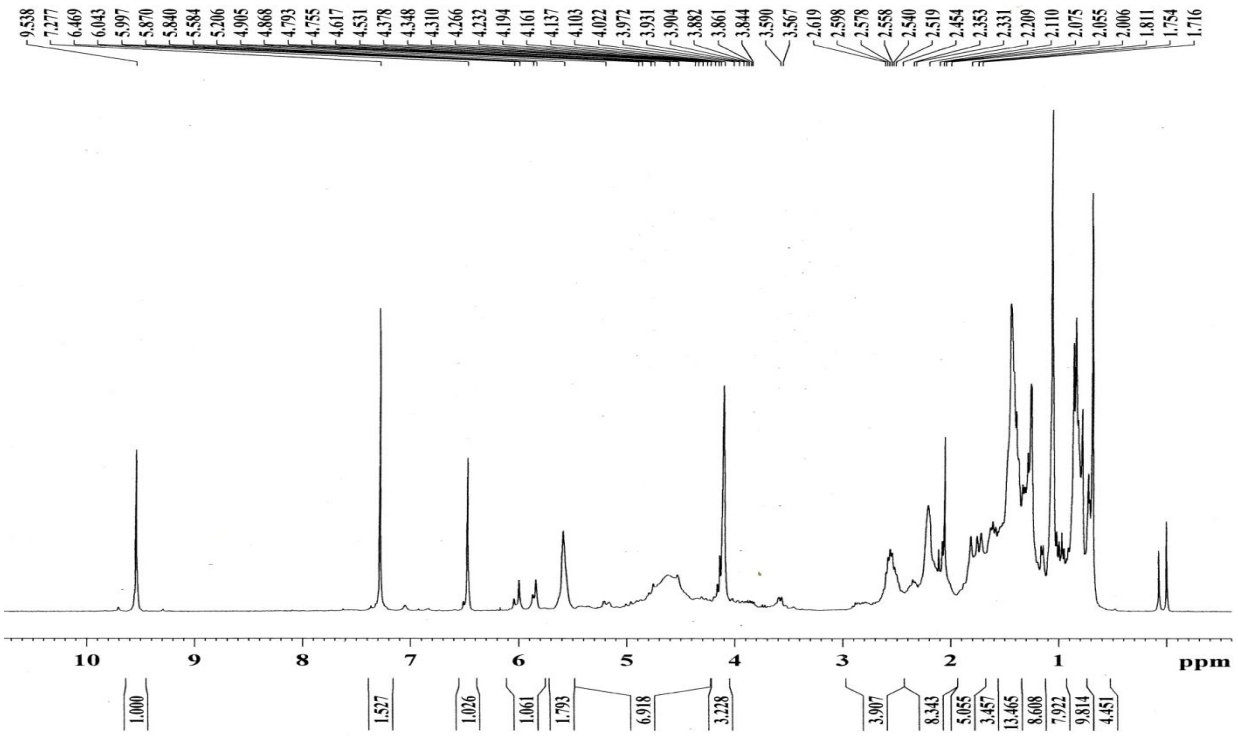


Fig. 60: ^{1}H NMR spectral data of 18-Hydroxy-16-oxocleroda-3, 13(14) E-dien-15-oic acid (Biotransformed products)

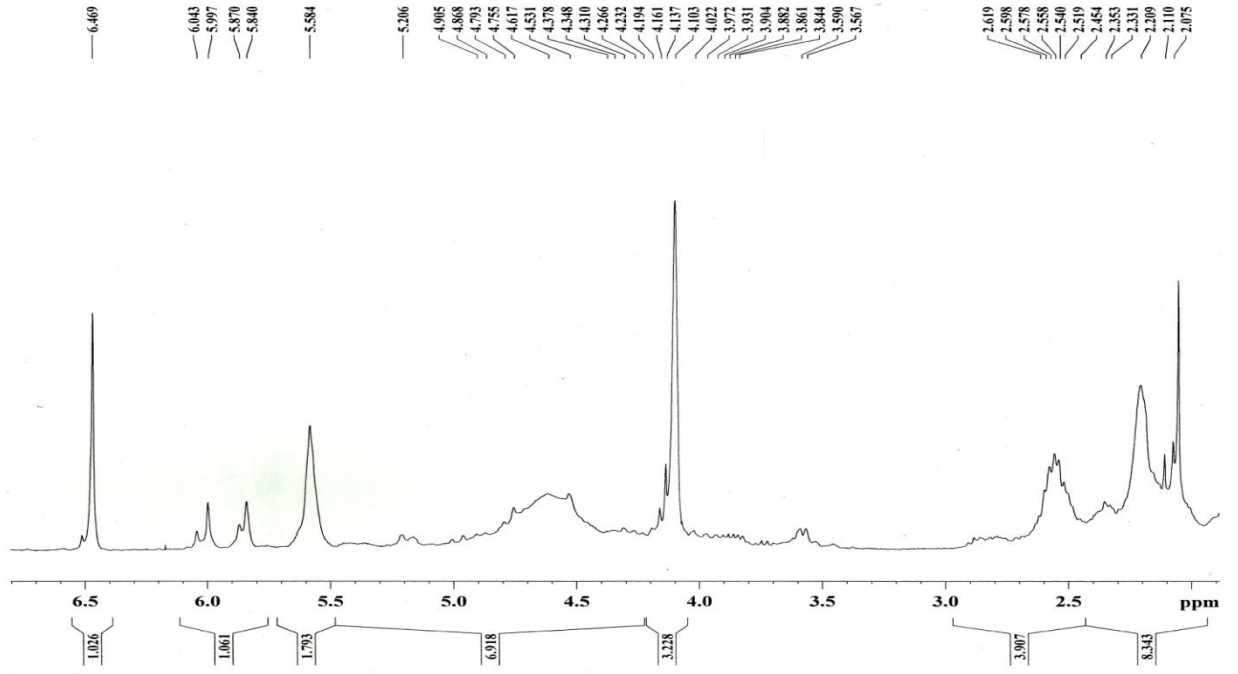


Fig. 61: ^{1}H NMR spectral data of 18-Hydroxy-16-oxocleroda-3, 13(14) E-dien-15-oic acid (Biotransformed products)

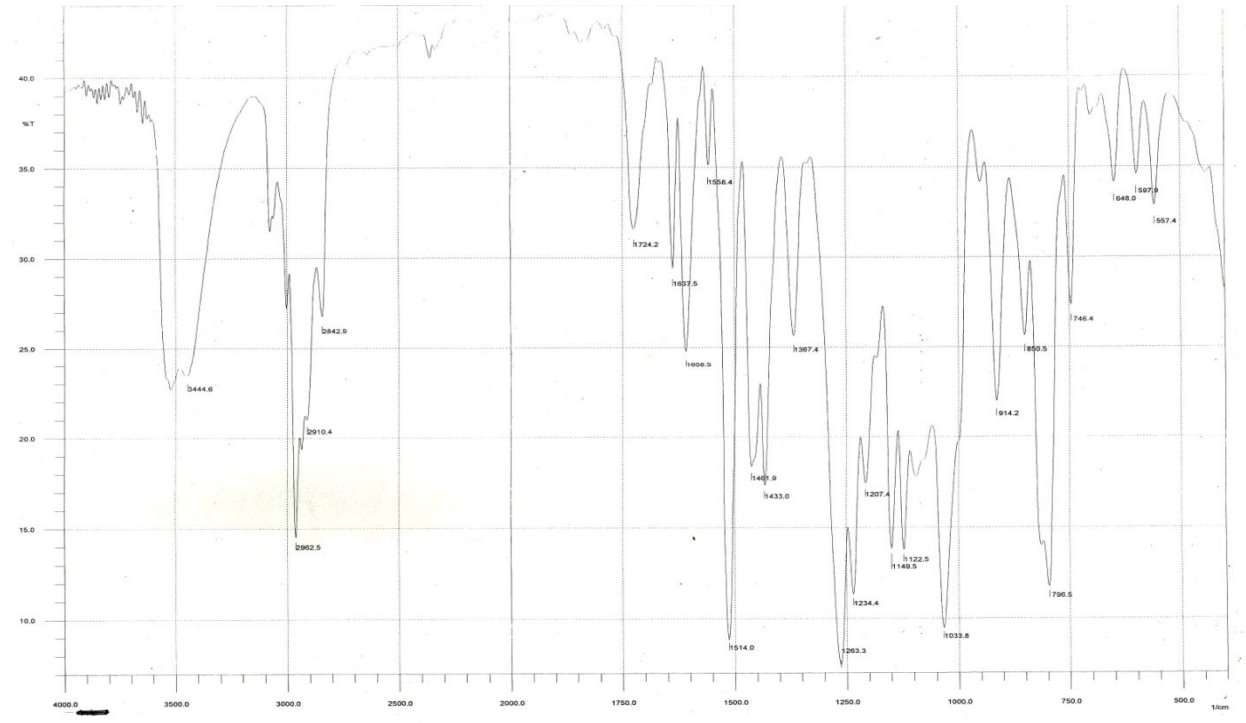


Fig. 62: IR spectral data of Eugenol

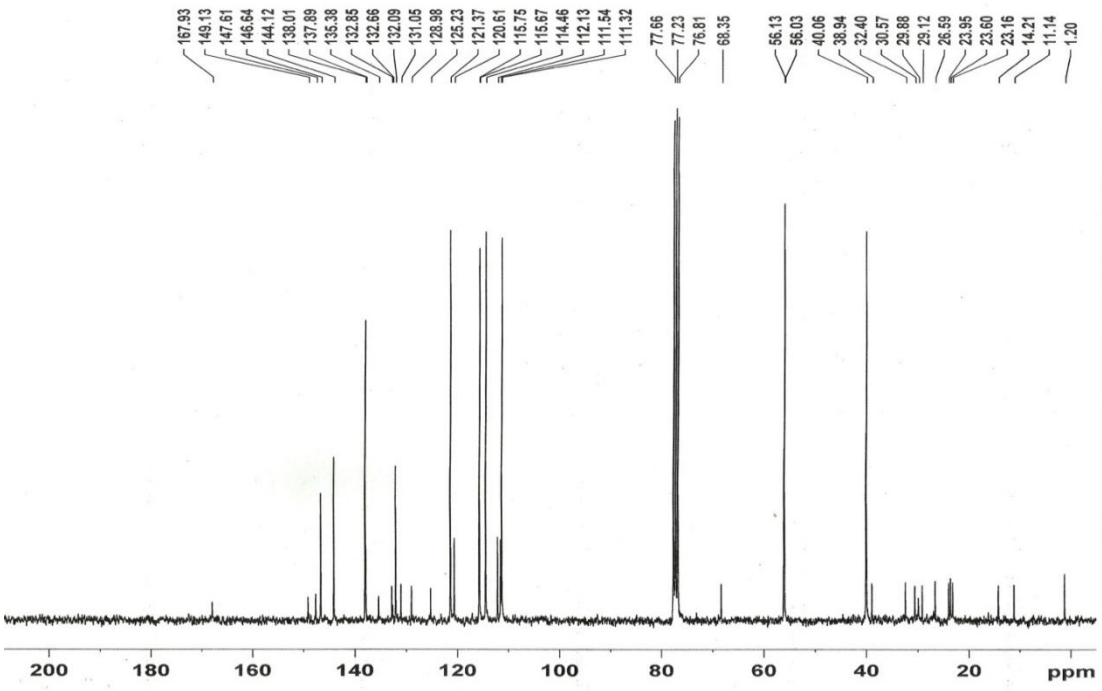


Fig. 63: ^{13}C NMR spectral data of Eugenol

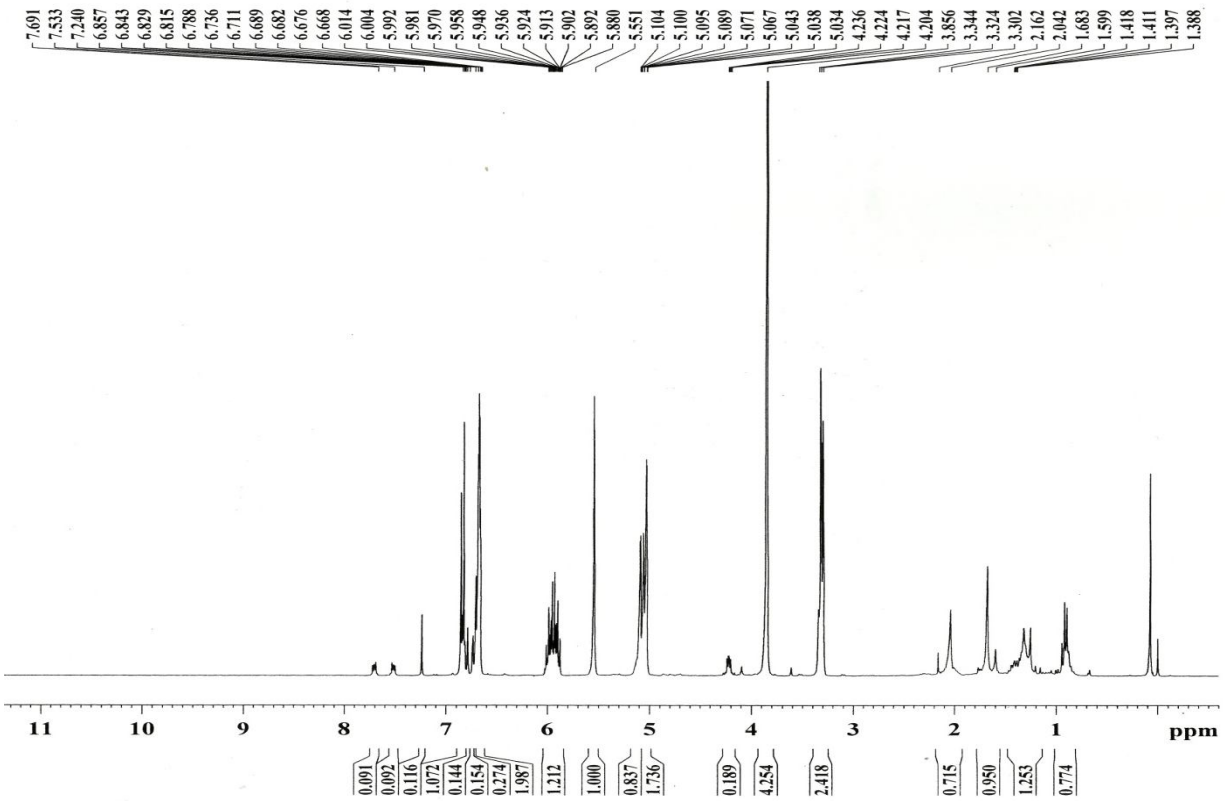


Fig. 64: ^{1}H NMR spectral data of Eugenol

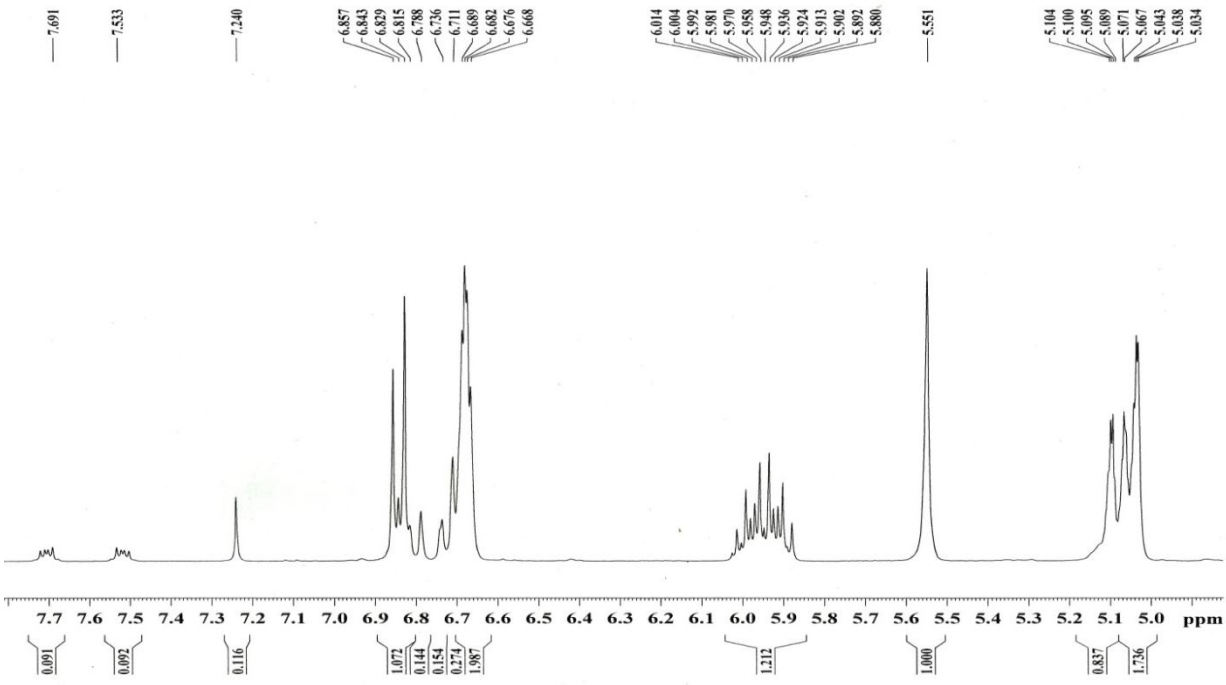


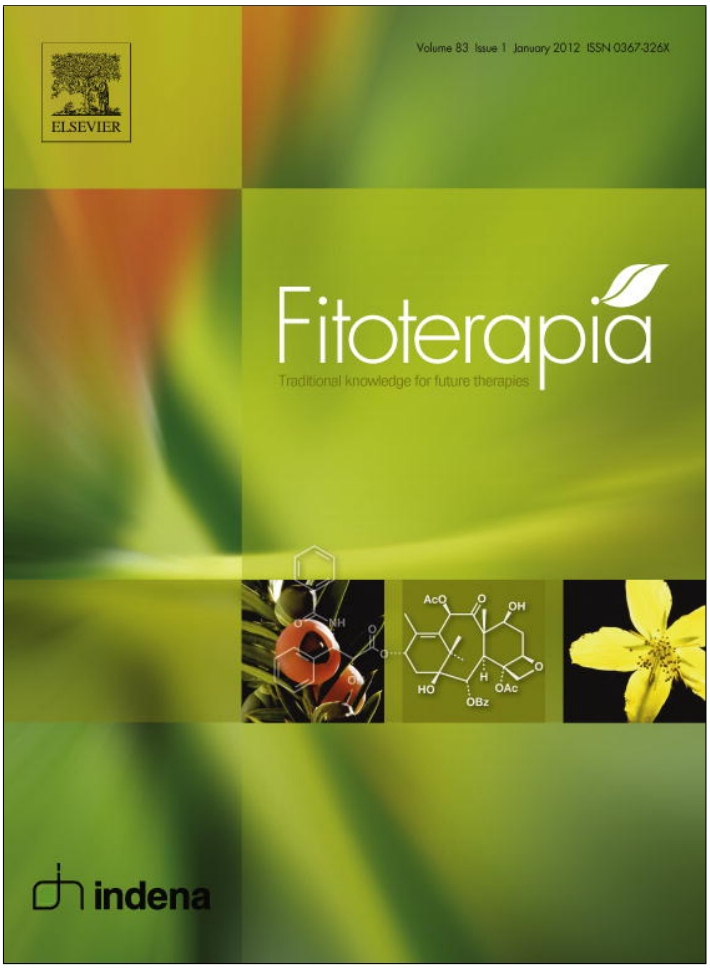
Fig. 65: ^{1}H NMR spectral data of Eugenol

LIST OF PUBLICATION

1. Ramashish Kumar, Aniruddha Saha, Dipanwita Saha (2012). A new antifungal coumarin from *Clausena excavata*. *Fitoterapia*. 83: 230-233.

2. Dipanwita Saha, Ramashish Kumar, S. Ghosh, M. Kumari and Aniruddha Saha (2012). Control of foliar diseases of tea with *Xanthium strumarium* leaf extract. *Industrial Crops and products.* 37: 376-382.

3. Ramashish Kumar, Aniruddha Saha, Dipanwita Saha (2014). Biotransformation of 16-oxacleroda-3-13E-dien-15-oic acid isolated from *Polyalthia longifolia* by *Rhizopus stolonifer* increases its antifungal activity. *Pharmaceutical Biology* (**Communicated**).



Fitoterapia 83 (2012) 230–233

Contents lists available at SciVerse ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

A new antifungal coumarin from *Clausena excavata*

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ARTICLE INFO

Article history:
Received 23 August 2011
Accepted in revised form 25 October 2011
Available online 10 November 2011

Keywords:
Clausena excavata
Rutaceae
Coumarin
Antifungal activity

ABSTRACT

A new γ-lactone coumarin, named as excavarin-A, showing antifungal activity was isolated from the leaves of *Clausena excavata* by bioassay guided fractionation method. The structure was elucidated by spectroscopic data analysis and identified as 7((2E)-4(4,5-dihydro-3-methylene-2-oxo-5-furanyl)-3-methylbut-2-enyloxy) coumarin. Minimum inhibitory concentration (MIC) was determined against fifteen fungal strains pathogenic against plants and human. The least MIC was recorded against the human pathogen, *Candida tropicalis* and the plant pathogens *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Antifungal activities against the human pathogens, *Aspergillus fumigatus* and *Mucor circinelloides* and plant pathogens, *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae*, *Fusarium oxysporum* and *Rhizopus stolonifer* were stronger than that of the standard antimicrobials.

1. Introduction

Clausena excavata Burm. f., a member of Rutaceae family, is widely distributed in India, China and Southeast Asia. The plant is a wild shrub; its leaves, twigs and root bark are used as folklore medicine [1,2] for the treatment of cold, malaria, abdominal pain, snake-bite, preliminary stage of AIDS and dermatopathy [3]. Diverse groups of bioactive chemical constituents are reported to be present in the different plant parts which are antibacterial [1], antifungal, antimycobacterial [4], antinociceptive [5], *in vivo* immunomodulating [6], anti HIV-1 [7] and insecticidal [8]. Phytochemical analyses in the past have revealed that *C. excavata* is a rich source of coumarins, carbazole alkaloid and limonoides [7,9–12]. The coumarins isolated from this plant have attracted attention due to its bioactive properties such as the furanone-coumarins named clauslactones A–J isolated from leaves exhibited tumor promotion inhibitory effects [9], nordentatin showed antibacterial properties [1] and a pyranocoumarin clausenidin isolated from roots displayed anti-HIV-1 activity [11].

In continuation with our interest in antifungal substances from plant sources, we performed preliminary studies with dichloromethane leaf extracts of *C. excavata* which revealed significant antifungal activity against a number of filamentous fungi and yeasts. The present study describes the isolation of a new coumarin with monoterpenoidal γ-lactone side chain (**1**) through bioassay-guided fractionation of leaf extract of *C. excavata*. Antifungal activity was determined by bioautography and micro-dilution assay. The activity was compared with the standard fungicide bavistin and the antibiotic nystatin.

2. Experimental

2.1. Plant material

The leaves of *C. excavata* were collected in October 2008 from the social forest sites within the campus of the University of North Bengal, located in the sub-Himalayan region of the state of West Bengal, India. A voucher specimen was deposited at the herbarium of the Taxonomy Division, Department of Botany, University of North Bengal (voucher number 9548). The plant material was dried at room temperature for 5 to 10 days and made powder.

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doi:10.1016/j.fitote.2011.11.003

2.2. Extraction and isolation

The dried and powdered leaves of *C. excavata* (200 g) were extracted with dichloromethane in a Soxhlet apparatus for 20 h at 35 °C. The extract was concentrated under reduced pressure to give a brown sticky solid (15 g) which was subjected to silica gel column chromatography. Elution with n-hexane, n-hexane: dichloromethane (3:1, 1:1, 1:3), dichloromethane, ethyl acetate: dichloromethane (3:1, 1:1, 1:3), ethyl acetate, methanol: ethyl acetate (3:1, 1:1, 1:3) and methanol gave thirteen fractions (F_1 to F_{13}). Based on activity tested through bioautography on TLC plates, fraction F_7 (2 g) was rechromatographed on a silica gel column, eluting with petroleum ether (200 ml) and EtoAc: hexane (2%, 5%, 10%, 15%, 20%, 25%, 30% and 35%) by which a total of 8 fractions (f_1 to f_8) were collected. The bioactive fraction f_7 and f_8 was fractionated into 40 sub-fractions (Sf_1 to Sf_{40}). Six sub-fractions (Sf_{30}–Sf_{35}) were pooled and crystallized with hexane–ethyl acetate to give compound **1** (110 mg). The crystallized compound was tested for antifungal activity by bioautography.

2.3. General experimental procedure

Commercial silica gel (60–120 mesh SRL, India) was used for column chromatography and precoated silica gel F254 aluminum plates (Merck) for TLC. Silica gel G and silica gel F254 were used for bioautography; the R_f value refers to silica gel TLC using hexane–ethyl acetate (60:40). Melting point was determined in open capillary tube and remains uncorrected. The UV spectrum was recorded in UV1/160A UV–visible spectrophotometer (Shimadzu) in ethyl acetate. The IR spectrum was detected in KBr in a Shimadzu-8300 FT–IR spectrophotometer. ESI-MS spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer, SAIF, Central Drug Research Institute, Lucknow. The NMR (^{1}H- and ^{13}C-) spectra were obtained in $CDCl_3$ from a Bruker AV 300 spectrometer with TMS as internal reference. (The chemical shifts of NMR are in δ ppm and *J* in Hz).

2.4. Excavarin-A, 7((2E)-4(4,5-dihydro-3-methylene-2-oxo-5-furanyl)-3-methylbut-2-enyloxy) coumarin

White needle shaped crystals (n-hexane–ethyl acetate), m.p. 104 °C; ESI-MS: m/z (%) = 327 [100, $(M+H)]^+$, 163 (24), 97 (4); UV ($CH_3COOC_2H_5$) λ_{max} nm: 319; IR λ_{max} (KBr) cm^{-1}: 1742, 1720, 1611; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H = 7.64 (1H, d, *J* = 9.6 Hz, H-4), 7.38 (1H, d, *J* = 8.4 Hz, H-5), 6.85 (1H, dd, *J* = 8.6, 2.1 Hz, H-6), 6.80 (1H, d, *J* = 2.1 Hz, H-8), 6.24 (1H, t, *J* = 3.0 Hz, H_a-10′), 5.65 (1H, t, *J* = 3.0 Hz, H_b-10′), 5.56–5.65 (1H, m, H-2′), 4.66–4.74 (1H, m, H-6′), 4.62 (2H, d, *J* = 6.3 Hz, H-1′), 3.06 (1H, ddt, *J* = 17.0, 7.8, 3.0 Hz, H_a-7′), 2.54 (1H, dd, *J* = 14.1, 7.5 Hz, H_a-5′), 2.38 (1H, dd, *J* = 14.3, 5.4 Hz, H_b-5′), 2.64 (1H, ddt, *J* = 17.0, 5.6, 3.0 Hz, H_b-7′), 1.83 (3H, s, H-4′), 6.25 (1H, d, *J* = 9.3 Hz, H-3); ^{13}C NMR (75 MHz, $CDCl_3$): δ_C = 161.2 (C-2), 113.1 (C-3), 143.4 (C-4), 128.8 (C-5), 122.3 (C-6), 161.8 (C-7), 101.6 (C-8), 155.8 (C-9), 112.6 (C-10), 65.0 (C-1′), 122.8 (C-2′), 136.5 (C-3′), 17.3 (C-4′), 45.7 (C-5′), 75.5 (C-6′), 33.1 (C-7′), 134.1 (C-8′), 170.0 (C-9′), 122.5 (C-10′).

2.5. Test organisms

The antifungal activity of the purified extracts was assessed against the fungal strains, *Aspergillus fumigatus* (MTCC 6594), *Aspergillus niger* (MTCC 2425), *Candida albicans* (MTCC 183), *Candida tropicalis* (MTCC 184), *Colletotrichum gloeosporioides* (ITCC 5446.02), *Cryptococcus curvatus* (MTCC 2698), *Curvularia eragrostidis* (ITCC 4150.2K), *Filobasidiella neoformans* (MTCC 1431), *Fusarium oxysporum* (ITCC 6246), *Lasiodiplodia theobromae* (ITCC 5446.02), *Mucor circinelloides* (MTCC 3944), *Rhizoctonia solani* (ITCC 5995.05), *Rhizopus stolonifer* (ITCC 6283), *Sclerotinia sclerotiorum* (ITCC 6094) and *Trichosporon cutaneum* (MTCC 1963). All the MTCC strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The ITCC strains were either obtained directly from the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi or they were isolated as plant pathogens from crop fields and their identities were authenticated by IARI, New Delhi.

2.6. Evaluation of antifungal activity by bioautography

Bioautography was done on glass-backed silica gel TLC plates by following published procedures [13,14] with some modifications. The plates were dried and activated at 70 °C for 45 min. Test extract (30 µl) which was concentrated and adjusted to 5 mg/ml was spotted on the activated TLC plates and developed in hexane: ethyl acetate (60:40). The developed chromatogram was air-dried until the solvent evaporated completely. For recording antifungal activity on TLC plate, spore suspension (10^5 spores/ml) of the test fungus in potato dextrose broth (15 g/l) was sprayed on the dried chromatogram and incubated in a moist chamber for 2–3 days at 28 °C. Inhibition of fungal growth on TLC plates, which appeared as clear zones in a background of mycelia and spores growing on the plate, indicated the presence of antifungal compound.

2.7. Minimum inhibitory concentration

Antifungal activity of the isolated compound was tested against 15 fungal strains by using the 96-well micro-titer plate assay. To determine the minimum inhibitory concentration (MIC), the active constituent was serially double diluted in ethanol (5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 mg/ml) and 50 µl of each of the different concentrations were pipetted into the wells of the micro-titer plate. The standard fungicide bavistin and the antifungal antibiotic nystatin were included for comparison. Next, ethanol was allowed to evaporate and then, each well was loaded with a mixture (100 µl) of sterile PDB and fungal inoculum. The inoculum was prepared from 4 to 8 day old fungal cultures in the form of either spore suspension (10^6 spores/ml) or fine mycelia fragment in PDB. Positive control wells contained inoculum and PDB only, without any test compound. A negative control set contained the test compound and PDB only. The plates were covered with plastic lid and incubated in a growth chamber at 28 °C. Fungal growth was monitored after 48 h by measuring absorbance at 600 nm using a microtiter-plate reader (Mios Junior, Merck). A zero hour reading was taken as blank. The MIC was considered as the lowest final concentration that did not record any growth.

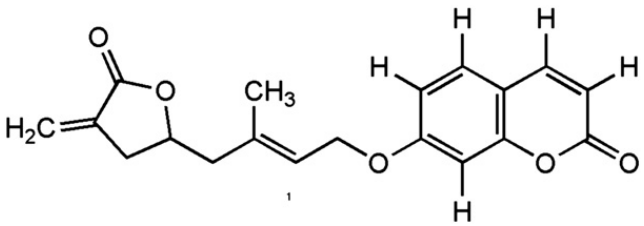

Fig. 1. Chemical structure of compound **1**.

3. Results and discussion

A novel γ-lactone coumarin named as excavarin-A (**1**) was isolated from *C. excavata* as colorless needle shaped crystals (Fig.1). The molecular formula was determined as $C_{19}H_{18}O_5$ by ESI-MS and molecular ion peak at m/z 327 $(M+H)^+$. IR spectrum showed the presence of a five-membered lactone ring (ν_{max}1742) and carbonyl group (1720 cm^{-1}). The structure of the compound was confirmed by the ^1H and ^{13}C NMR data compared with published spectroscopic data [2,9,11]. The ^1H NMR spectrum showed characteristic signals at δ_H 6.25, 7.64 (each 1H, d, $J=9.6$ Hz), 7.38(1H, d, $J=8.4$ Hz) and 6.85 (1H, dd, $J=8.6$, 2.1 Hz) for H-3, H-4, H-5 and H-6 respectively indicating the presence of coumarin nucleus. The side chain of this coumarin structure was elucidated by comparing its ^1H and ^{13}C NMR spectra with that of the known compound clauslactone-E (**2**) [9]. The ^1H NMR signal δ_H 4.62 (2H, d, $J=6.3$ Hz, H-1′), 5.62–5.65 (1H, m, H-2′), 1.83 (3H, s, H-4′), 2.54 (1H, dd, $J=14.1$, 7.5 Hz, H_a-5′) and 2.38 (1H, dd, $J=14.3$, 5.4 Hz, H_b-5′) and ^{13}C NMR peak at δ_C 65.0, 122.7, 136.5, 17.3 and 45.7 indicated the presence of side chain of $-OCH_2-CH-C(CH_3)-CH_2-$. Further, a carbonyl carbon signal of lactone ring at δ_C 170.0 and a methylene signal at δ_C 122.5 and δ_H 6.24, 5.65 (1H, t, $J=3.0$ Hz) indicated that these moieties were attached to the side chain of lactone ring. In ESI-MS spectral data of compound **1**, cleavage of bond between oxygen atom at C-7 and side chain resulted in the formation of stable 7-hydroxycoumarin and at m/z 163. Additionally the α-methylene γ-lactone ring ion gave a peak at m/z 97. The significant difference between compounds **1** and **2** is absence of hydroxyl group at position C-8 (^{13}C NMR signal at δ_C 101 instead of δ_C 133.3) [9]. It also differed from excavatin D (**3**), which did not contain methylene group attached to the lactone ring [11] (Fig. 2). Based on all the above data, it was concluded that the structure of the isolated coumarin is as depicted in **1**. The nearest structure was reported in literature is **2** [9].

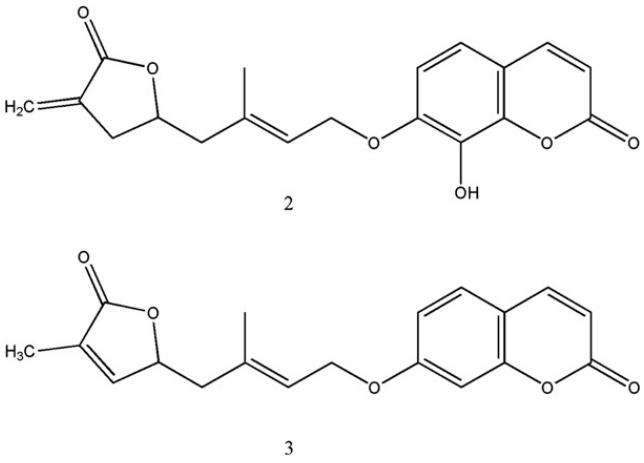

Fig. 2. Structure of compounds **2** and **3**.

Bioactivity of the extract was monitored by bio-autography; the presence of antifungal compound was evident by the appearance of a clear zone of inhibition at R_f 0.34. The purified compound was evaluated for its antifungal activity against fifteen fungal strains by the 96-well microtiter plate assay and activity was compared to the standard fungicides, bavistin and nystatin. Test compounds were serially diluted and subsequently mixed with potato dextrose broth and inoculum in microtiter plates to obtain eight dilutions (2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 mg/ml). The result of MIC evaluation is given in Table 1.

Antifungal activity of the isolated compound was found to be stronger than that of the standard antibiotic nystatin against the clinically important pathogens, *A. fumigatus*, *C. tropicalis* and *M. circinelloides*. The plant pathogenic fungi, *C. gloeosporioides*, *C. eragrostidis*, *F. oxysporum*, *L. theobromae*, *R. solani*, *R. stolonifer* and *S. sclerotiorum* were more sensitive toward the isolated compound than the standard fungicide bavistin. *R. solani* and *F. oxysporum* were found to be the most sensitive strains with an MIC value less than 0.019 mg/ml. Fungicidal action against *C. curvatus* and *T. cutaneum* was observed to be similar to the standard antibiotic. *A. niger* and *A. fumigatus* were found to be most resistant (MIC 0.625 mg/ml). The antagonistic potential of the isolated coumarin was found to be considerably strong which may be due to the α-methylene-γ-lactone moiety present in the side chain. Such systems are implicated in bioactivity in the sesquiterpene lactones [15]. However, α,β-unsaturated carbonyl moiety makes the sesquiterpene lactones reactive toward

Table 1
Antifungal activity of compound (**1**) purified from leaf extracts of *Clausena excavata*.

Tested fungal pathogens	Strain no.	Minimum inhibitory concentration (MIC) in mg/ml		
		Compound **1**	Bavistin	Nystatin
Human pathogens				
Aspergillus niger	MTCC 2425	0.625	–[a]	0.156
Aspergillus fumigatus	MTCC 6594	0.625	–	1.25
Candida albicans	MTCC 183	0.078	–	0.039
Candida tropicalis	MTCC 184	0.039	–	0.625
Cryptococcus curvatus	MTCC 2698	0.078	–	0.078
Filobasidiella neoformans	MTCC 1431	0.078	–	<0.019
Mucor circinelloides	MTCC 3944	0.078	–	0.156
Trichosporon cutaneum	MTCC 1963	0.078	–	0.078
Plant Pathogens				
Colletotrichum gloeosporioides	ITCC 5446.02	0.039	0.078	–
Curvularia eragrostidis	ITCC 4150.2 K	0.312	1.25	–
Fusarium oxysporum	ITCC 6246	0.019	2.5	–
Lasidiplodia theobromae	ITCC 4151.2K	0.039	0.078	–
Rhizoctonia solani	ITCC 5995.05	<0.019	0.039	–
Rhizopus stolonifer	ITCC 6283	0.019	0.039	–
Sclerotinia sclerotiorum	ITCC 6094	<0.019	0.078	–

[a] '–' = Not tested.

thiol groups of proteins [16]. Therefore, further studies on probable toxicity of excavarin A are necessary before developing it as a therapeutic agent. Anisocoumarin A and B isolated from leaves and twigs of *C. anisum-olens* which did not contain the α-methylene-γ-lactone moiety, failed to show antifungal activity against *Candida albicans*, *C. tropicalis* and *C. krusei* [17]. The study presents the isolation of a new compound named as excavarin A with strong antifungal activity which may be utilized as an antifungal agent against pathogenic fungi of both clinical and agricultural importance.

Acknowledgments

The authors wish to thank Prof. B. Basu and Mr. Jayanta Das of the Department of Chemistry, University of North Bengal for helpful suggestions during spectroscopic analysis. RK is thankful to Indian Council for Medical Research for granting Research Fellowship. Financial assistance received from CSIR, New Delhi is gratefully acknowledged.

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Industrial Crops and Products 37 (2012) 376–382

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Control of foliar diseases of tea with *Xanthium strumarium* leaf extract

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ARTICLE INFO

Article history:
Received 12 July 2011
Received in revised form
20 December 2011
Accepted 21 December 2011

Keywords:
Xanthium strumarium
Xanthatin
Antifungal activity
Herbal fungicide
Camellia sinensis

ABSTRACT

Tea industry forms the backbone of economy in the sub-Himalayan agro-climatic region of north east India where it is normally grown extensively in large plantations. The study was aimed to develop a bio-rational alternative to the harmful chemical fungicides for controlling foliar fungal diseases which limit tea production. An antifungal compound from benzene extract of adult leaves from *Xanthium strumarium* L. was purified and characterized and the *in vitro* antifungal activity was evaluated against a broad spectrum of plant pathogens. The sesquiterpene lactone which was purified by bioassay guided fractionation using repeated column chromatography and thin layer chromatography was identified as xanthatin by ultraviolet, infrared and nuclear magnetic resonance spectroscopic analysis. The minimum inhibitory concentration of the isolated compound tested by microdilution method ranged from <0.0097 to 0.325 mg ml^{-1} against 8 tea pathogens and 12 other plant pathogens. The most susceptible fungi were *Colletotrichum camelliae*, *Curvularia eragrostidis* and *Rhizoctonia solani* among tea pathogens and *Colletotrichum gloeosporioides*, *Fusarium moniliforme* and *Rhizopus stolonifer* among other tested pathogens. Subsequently the *in vivo* antifungal activity was tested in detached leaves against four foliar tea pathogens, *C. camelliae*, *C. eragrostidis*, *Lasidiplodia theobromae* and *Pestalotiopsis theae*. The purified compound completely inhibited lesion development at 0.1 mg ml^{-1} concentration against *C. camelliae* and *C. eragrostidis*. The purified compound and the crude extract were further tested for their ability to control foliar diseases in young tea plants of two clonal varieties, TV-18 and TV-30 in the greenhouse. The water insoluble extracts emulsified in water effectively controlled brown blight (*C. camelliae*), leaf spot (*C. eragrostidis*), grey blight (*P. theae*) and leaf blight (*L. theobromae*). Highest percentage efficacy of disease control was exhibited against leaf spot followed by brown blight. The disease control efficacy was comparable to the fungicide bavistin. The results show that the adult leaves of *X. strumarium* may be used as a source of botanical fungicidal preparation for controlling foliar fungal diseases of tea.

1. Introduction

Tea (*Camellia sinensis* L. (O) Kuntze) is the most popular beverage which is consumed throughout the globe. It is extensively grown in the sub-Himalayan agro-climatic zone of north and north-east India and a large population of people of this entire region is dependent on the tea industry for their livelihood (Dasgupta et al., 2005). It is also grown in several places in southern India, Srilanka and China. Foliar diseases are a major threat to the production of adequate and healthy tea leaves which requires the use of large amount of chemical fungicides (Singh, 2005). Although chemical pesticides are effective in controlling diseases, but they have now become unpopular due to several reasons, such as residual toxicity (Seth et al., 1998), non target environmental impacts, growing cost of pesticides, modified safety regulations (Gerhardson, 2002; Compant et al., 2005), development of pathogen resistance (Van den Bosch and Gilligan, 2008) and ineffectivity of chemicals in fastidious cases (Compant et al., 2005). It is evident from past reports that plant products have huge potential as antimicrobial agents (Cowan, 1999). Some of these are useful in agriculture as they are capable of inhibiting the growth of fungal phytopathogens. They are regarded as environmentally safer and preferred to synthetic fungicides for the purpose of crop protection (Isman, 2000). Moreover they provide a better affordable alternative to pharmaceutical commodities in the developing countries (Tegegne et al., 2008).

A programme of screening of plant species for antifungal activity was undertaken in our laboratory (Saha et al., 2005a) and of the several Indian plant species that possessed antifungal properties against pathogens of tea, *Xanthium strumarium* L. (English: Cocklebur, Family: Asteraceae) was found to be the most potent. *X. strumarium* is a common weed found abundantly throughout the world. It is an annual herb (20–150 cm tall) with hairy stem which is frequently branched resulting in a bushy appearance. The plant grows naturally on riversides but readily spreads from natural

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doi:10.1016/j.indcrop.2011.12.030

habitats and establishes weedy populations in urban waste areas (Bozsa and Oliver, 1990; Islam et al., 2009). *X. strumarium* extracts are reported to possess several beneficial biological properties, such as antihelmintic (Sharma et al., 2003), anticancerous (Ramirez-Erosa et al., 2007), antibacterial (Kanauchi et al., 1999), antileishmanial (Lavault et al., 2005), antimalarial (Joshi et al., 1997), antitrypansomal (Talakal et al., 1995), anti-inflammatory (Kim et al., 2005), antiulcerogenic (Favier et al., 2005), antinociceptive (Kim et al., 2005) and antifungal (Lavault et al., 2005). However, reports on antifungal activity against phytopathogens are limited (Kim et al., 2002; Ginesta-Peris et al., 1994) and the utilizable potential of *X. strumarium* extracts as agricultural fungicides remains unexplored.

The objective of this study was to test the efficacy of crude and purified extracts of *X. strumarium* for controlling foliar fungal diseases of tea. First we conducted a study on the antifungal activity of *X. strumarium* extracts through bioassay-guided purification of the active compound and determined the spectrum of antifungal activity of the purified compound against 20 phytopathogens of economic importance. Subsequently, the compound was tested *in vivo* against four common foliar fungal pathogens of tea in terms of minimum inhibitory concentration (MIC) in detached leaf assay. Finally the control of the diseases was studied in young tea plants using a formulation of *X. strumarium* extract in both crude and purified form under green house conditions.

2. Materials and methods

2.1. Plant material

X. strumarium leaves in adult stage were collected from large bushes in sub-Himalayan region of West Bengal, India in June–July 2008 where it grows abundantly at road side and waste places. A voucher specimen was deposited in the herbarium (Acc. No. 9549) of Department of Botany, University of North Bengal. The plant species were identified by Prof. A.P. Das of the Department of Botany, University of North Bengal.

Fresh tea leaves of TV-18 and TV-30 varieties that differed in their susceptibilities to fungal pathogens were collected from the departmental tea garden when required. TV-18 is recorded as highly susceptible while TV-30 is recorded as moderately susceptible to different foliar pathogens (Chakraborty and Saha, 1994; Chakraborty et al., 1995; Dasgupta et al., 2005).

2.2. Fungal culture

The plant extract was tested against 20 fungal phytopathogens of which *Colletotrichum camelliae*, *Curvularia eragrostidis*, *Lasidiplodia theobromae*, *Pestalotiopsis theae*, *Poria hypobrunea*, *Sphaerostilbe repens*, *Rhizoctonia solani* and *Ustulina zonata* are pathogens of tea. *P. hypobrunea*, *S. repens* and *U. zonata* were provided by Tea Research Association, Tocklai Experimental Station, Jorhat, Assam, India. *P. theae* and *C. camelliae* strains were obtained from UPASI Tea Research Foundation, Tea Research Institute, Valparai, Coimbatore, India. Among the other 12 pathogens, *Rhizopus stolonifer*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Pythium aphanidermatum*, *Macrophomina phaseolina* and *Sclerotinia sclerotiorum* were obtained from the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. All other pathogens viz. *C. eragrostidis*, *L. theobromae*, *R. solani*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium equiseti*, *Fusarium moniliforme*, *Fusarium solani* and *Aspergillus niger* were isolated in the laboratory and their identities were authenticated by IARI, New Delhi. The pathogenicities of isolated strains were confirmed by verification of Koch's postulates. All fungal cultures were maintained in the laboratory in potato dextrose agar (PDA) slants.

2.3. Extraction and purification

Fresh leaves were thoroughly washed with distilled water and shade dried at room temperature for 5–10 days. The dried leaves (500 g) were ground to moderately fine powder (1 mm) and soxhlet extracted with benzene (1500 ml) for 15 h at 35 °C. The extract was concentrated to dryness under vacuum in a rotary evaporator (Eyela, Japan). The residue (40 g) obtained as a gummy solid, was considered as crude extract. For further purification, the crude extract was mixed with 20 g silica gel and dried at 40 °C. The mixture was then loaded onto silica gel column (60 × 3 cm) and eluted with hexane, hexane: dichloromethane (3:1, 1:1, 1:3), dichloromethane, dichloromethane: ethyl acetate (3:1, 1:1, 1:3), ethyl acetate, methanol: ethyl acetate (3:1, 1:1, 1:3) and methanol by which 13 fractions (F_1 to F_{13}) of 100 ml each were obtained. Each fraction was concentrated under vacuum and bioassayed following bioautography method (Saha et al., 2005b). Fraction F_8 (0.5 g) which showed antifungal activity was rechromatographed on silica gel column using hexane (200 ml) and hexane: EtOAc (98:2, 95:5, 90:10, 85:15, 80:20, 75:25 and 70:30) as eluting solvent. Eight fractions were collected and each was monitored by bioautography as stated before. Two fractions showing antifungal activity were rechromatographed on silica gel and further fractionated and bioassayed and the bioactive fraction (approx. 30 mg) was analysed by UV–Vis, IR and NMR (^{1}H- and ^{13}C-) spectroscopy.

2.4. Identification of antifungal compound

The purity of the fraction with antifungal activity was checked by TLC (Merck aluminum plates coated with silica gel 60 F_{254}) after running in hexane: ethyl acetate: methanol (60:40:1). The plates were developed by UV and by spraying with chemical developer. Identification of purified compound was accomplished by comparison of the spectral data with the previously described compound. UV–Vis spectrum was measured on a UV1/160A/UV–Visible Recording Spectrophotometer (Shimadzu, Japan). IR spectrum was recorded on a Shimadzu-8300 FTIR spectrophotometer as a film on KBr pellets. ^{1}H- and ^{13}C-NMR spectra were determined on a Bruker-AV 300 spectrometer operating respectively at 300 MHz and 75 MHz and using TMS as internal standard.

2.5. In vitro antifungal activity of the purified constituent

The active constituent was serially double diluted in ethanol to obtain nine different dilutions whose concentrations were 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 and 0.0195 mg ml^{-1}. A 50 μl aliquot of each of the different concentrations were pipetted into the wells of a 96-well micro-titre plate. Ethanol was allowed to evaporate from the wells following which 100 μl of fungal inoculum in the form of either spore suspension (10^6 spores ml^{-1} in sterile PDB) or fine mycelia fragment was added to each well. Positive control set was without test compound (ethanol only) while in the negative control, sterile PDB replaced the inoculums. The plates were covered with plastic lid and incubated in a growth chamber at 28 °C for 48 h. Fungal growth was recorded by measuring absorbance at 600 nm using a microtitre-plate reader (Mios Junior, Merck). A zero hour reading was taken as blank. The MIC was considered as the lowest final concentration that did not record any growth.

2.6. Evaluation of antifungal activity of purified constituent and determination of MIC in vivo

Leaves (5–7 cm long) were detached from third or fourth node of mature tea bushes of TV-18 and TV-30 varieties, cleaned thoroughly and placed on blotting paper moistened with sterile distilled water. The blotting paper was laid in trays and covered with a glass lid to form humid chambers. Thirty microlitre of active constituent of *X. strumarium* (0.025–12.8 mg ml^{-1} in ethanol) was placed centrally as spots on the adaxial surface of each leaf on both sides of the midrib. The standard fungicide, bavistin was included in the experiment as positive control. For inoculation, two wounds (light scratch of 2 mm length) were made with a sterile sharp needle in place of the spots after evaporation of ethanol. For inoculum preparation, the fungi *C. camelliae*, *L. theobromae*, *P. theae* and *C. eragrostidis* were grown separately in PDA plates for five days at 28 °C. Mycelial plugs collected from the cultures were placed on the wounds of the tea leaves and covered with sterile cotton. A set of leaves which received ethanol instead of the purified constituent was considered as untreated-inoculated control. Trays were kept at 28 °C in a growth chamber with 12 h photoperiod. The experiment was repeated thrice. Lesion diameters were measured after 24, 48 and 72 h and mean lesion diameter was calculated for each pathogen. The results of three experiments were averaged. The lowest concentration that failed to induce lesion was considered as MIC.

2.7. Control of foliar fungal diseases in tea plantlets

2.7.1. Preparation of crude and purified extract formulations

Fresh leaves of *X. strumarium* were collected and washed thoroughly with sterile distilled water and dried at room temperature. The dried leaves were ground, weighed and extracted with benzene in a soxhlet apparatus. The solvent was evaporated at 40 °C in a Rotary Vacuum Evaporator (N-1000, Eyela, Japan). The resulting crude extract (4 mg) and the purified compound (2 mg) were dissolved separately in 500 μl of acetone and mixed with 40 μl of soyabean oil and 60 μl of Tween 80. Then 900 μl of distilled water was added to each mixture and both were allowed to stand until acetone was evaporated (Wiwattanapatapee et al., 2009). These emulsified extracts were used for testing disease reduction in greenhouse. A separate blank emulsifying mixture was prepared in the same proportions to serve as control.

2.7.2. Disease control using X. strumarium extract

For studying the level of protection that may be obtained by the extract preparations, well established young tea plants (7–10 leaves) raised from clonal cuttings of TV-18 and TV-30 varieties were transferred to pots (10 cm × 15 cm) containing a mixture of garden soil and farm yard manure (2:1). Experimental plants were sprayed by the formulated extracts on the upper leaf surface until runoff. Plants sprayed with the blank emulsifying mixture and untreated plants served as control. A fungicide control was included where plants were sprayed with bavistin (2 mg ml^{-1}). Extracts were sprayed at 1 h, 12 h, 24 h and 48 h prior to inoculation with the pathogen. The experimental and control plants were inoculated by spraying a pure conidial suspension (containing 0.05% tween 20) of foliar fungal pathogens four times at two day interval. The conidial suspension was prepared in sterile distilled water (1×10^6 conidia ml^{-1}) from 10- to 12-day-old PDA cultures of each of the pathogens *P. theae*, *C. eragrostidis*, *L. theobromae* and *C. camelliae* as described earlier (Saha et al., 2005a) and sprayed separately in separate experimental sets. The plants were kept in a transparent and perforated polythene chamber for maintaining high humidity and aeration during the inoculation period. Subsequently the plants were removed from the chamber and grown with normal soil surface watering. Humidity was maintained by spraying sterile distilled water at intervals. The pots were arranged in a randomized block design, with five replicates per treatment for each pathogen. The whole experiment was conducted thrice and the results of individual sets were pooled together and averaged. Assessment for disease development was done after 4, 8, 12, 16 and 20 days of the last inoculation.

2.7.3. Disease assessment

Disease development was assessed following the method of Dasgupta et al. (2005). The lesions were graded according to their size as very small (1–2 mm), small (2–4 mm), medium (4–6 mm) and large (>6 mm) and respective values of 0.1, 0.25, 0.5 and 1.0 assigned to them. Number of lesions on each group was multiplied by its assigned value. Disease index of every plant was calculated as the sum total of such values for all its leaves. Results were computed as the mean of observations of all plants in the respective experimental sets and expressed as disease index per plant. Percent efficacy of disease control (PEDC) was calculated using the formula: PEDC = [(disease index in control – disease index in experimental plants)/disease index in control] × 100 (Purkayastha et al., 2010).

2.8. In vivo assessment of extract phytotoxicity

In every experiment there was a separate set of treated-uninoculated leaves/plants in which only extract was put in drops or sprayed but inoculation was not done, for observing visibility of any tissue necrosis.

2.9. Statistical analysis

Data were analysed by ANOVA using Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois. Differences were compared by computing the Critical Difference (CD) at 5% level.

3. Results

3.1. Evaluation of antifungal activity in vitro

The compound purified from *X. strumarium* leaf extract exhibited antifungal activity against all the 20 fungal strains when tested by the 96-well microtiter plate assay. Test compounds were serially diluted and subsequently mixed with potato dextrose broth and inoculums in microtiter plates to obtain nine dilutions (2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, 0.0097 mg ml^{-1}). MIC values recorded against individual fungi are summarized in Table 1. Among the tea pathogens, the MIC was lowest against *C. camelliae* (<0.0097 mg ml^{-1}), followed by *C. eragrostidis* and *R. solani* (both 0.0097 mg ml^{-1}), and *L. theobromae* and *S. repens* (both 0.039 mg ml^{-1}). *P. theae* and *U. zonata* were completely inhibited at 0.078 mg ml^{-1}. The MIC was highest against *P. hypobrunnea* (0.156 mg ml^{-1}). Among other phytopathogens, those exhibiting maximum susceptibility were *C. gloeosporioides*, *R. stolonifer* and *F. moniliforme* (MIC: <0.0097 mg ml^{-1}) while *S. rolfsii* was the most tolerant (MIC: 0.312 mg ml^{-1}).

3.2. Structural identification of the antifungal compound

Repeated bioassay guided chromatography in Silica gel columns led to the isolation of a sesquiterpene lactone (Winters et al., 1969). The UV spectrum of this bio-active compound, taken in EtOH solution showed a strong absorbance with λ_{max} at 277 nm indicating presence of unsaturated carbonyl function. The infrared absorption spectrum was run in neat and ν_{max} were observed mainly at 1758, 1750, 1660 cm^{-1}, which revealed the presence of carbonyl groups

Table 1
Minimum inhibitory concentration (MIC) of the compound purified from benzene extract of *Xanthium strumarium* leaves against plant pathogens.

Fungus	Strain No.	*X. strumarium* purified constituent (mg ml^{-1})
Alternaria alternata	7063.08	0.156
Aspergillus niger	7958.10	0.078
Curvularia eragrostidis[a]	4150.2k	0.0097
Colletotrichum camelliae[a]	CC-01	<0.0097
Colletotrichum gloeosporioides	5446.02	<0.0097
Fusarium equiseti	6566.07	0.156
Fusarium moniliforme	7344.09	<0.0097
Fusarium oxysporum	6246	0.078
Fusarium solani	4999	0.019
Lasiodiplodia theobromae[a]	5446.02	0.039
Macrophomina phaseolina	5519	0.156
Pestalotiopsis theae[a]	PT-02	0.078
Poria hypobrunnea[a]	PH-01	0.156
Pythium aphanidermatum	4746	0.156
Rhizopus stolonifer	6283	<0.0097
Rhizoctonia solani[a]	5995.05	0.0097
Sclerotinia sclerotiorum	6094	0.039
Sclerotium rolfsii	6415	0.325
Sphaerostilbe repens[a]	SR-09	0.039
Ustulina zonata[a]	US-12	0.078

[a] Pathogens of tea.

(corresponding to keto and lactone carbonyl) and C=C double bond. The well defined absorption at 1758 cm^{-1} indicated presence of α-methylene-γ-lactone moiety (Olivaro and Vazquez, 2009). The ^{1}H-NMR spectrum of the bio-active sample in $CDCl_3$ showed signals at δ 1.22 (3H, d, J = 15.0); 2.29 (3H, s); 4.65 (1H, ddt, J = 2.5, 9.0, 13.0); 5.57 (1H, d, J = 3.0); 6.20 (1H, d, J = 1.2); 6.31 (1H, d, J = 3.0) and 6.98 (1H, d, J = 15.5) (δ in ppm and J in Hz). The IR and ^{1}H-NMR spectra of the compound were similar to xanthatin (Ginesta-Peris et al., 1994; Kanauchi et al., 1999). The ^{13}C-NMR spectrum showed a methylene signal at δ_C 122.6 (δ_H 6.31 and 5.57) which was similar to that obtained by Kim et al. (2002). Thus, combination of all spectral data followed by analyses, led us to conclude that the bio-active component possessing antifungal activity was xanthatin (Fig. 1).

3.3. Evaluation of antifungal activity in vivo

The foliar pathogens *C. eragrostidis*, *C. camelliae*, *L. theobromae* and *P. theae* were selected for studying the degree of antifungal activity of the compound isolated from *X. strumarium in vivo* in detached tea leaves. The extracted compound was found to be effective *in vivo* as evident from a progressive reduction in lesion diameter with increasing extract concentration up to 72 h against all the tested pathogens (Fig. 2). MIC values recorded after 72 h against individual fungi are summarized in Table 2. These concentrations were in general found to be lower when tested in detached leaves of TV-30 than in TV-18 variety. *C. camelliae* and *C. eragrostidis*

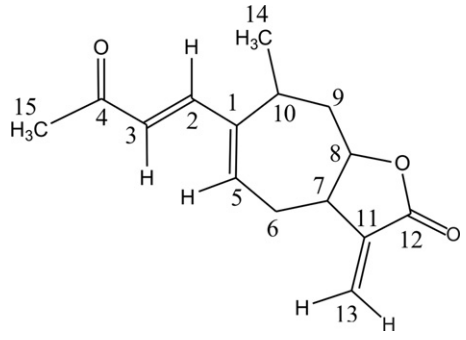

Fig. 1. Chemical structure of the antifungal compound purified from benzene extract of *Xanthium strumarium* leaves.

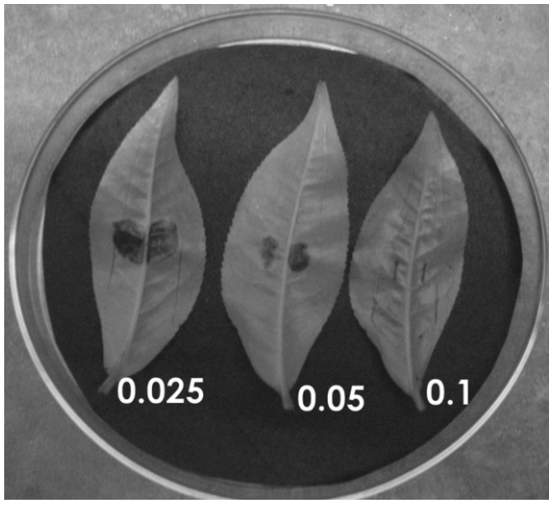

Fig. 2. *In vivo* antifungal activity of purified compound from *Xanthium strumarium* leaf extract against *Colletotricum camelliae* causing brown blight in tea. The applied concentrations are shown in mg ml^{-1}.

Table 2
Minimum inhibitory concentrations (MIC) of the compound isolated from *Xanthium strumarium* leaf extracts determined *in vivo* in detached leaves of two tea cultivars TV-18 and TV-30 against four common foliar fungal pathogens.

Fungus	TV-18		TV-30	
	X. strumarium purified constituent (mg ml^{-1})	Bavistin (mg ml^{-1})	*X. strumarium* purified constituent (mg ml^{-1})	Bavistin (mg ml^{-1})
Colletotrichum camelliae	0.2	0.4	0.1	0.2
Curvularia eragrostidis	0.2	>12.8	0.1	>12.8
Lasiodiplodia theobromae	0.8	0.4	0.4	0.4
Pestalotiopsis theae	1.6	0.4	0.4	0.4

were found to be most susceptible as both showed the lowest MIC value of 0.1 mg ml^{-1} in TV-30 and 0.2 mg ml^{-1} in TV-18. MIC was highest against *P. theae* (0.4 mg ml^{-1} in TV-30 and 1.6 mg ml^{-1} in TV-18). In control leaves which were either untreated or treated with emulsifying mixture and inoculated with the test pathogens, necrotic spots were visible at the wound site after 24 h which gradually increased until 72 h after inoculation. In leaves treated with the purified product at concentrations lower than MIC and inoculated with pathogen, necrotic spots were visible only after 48 h and the lesion development was slower than untreated leaves (data not shown). The MIC values of the synthetic fungicide bavistin and the test compound were approximately close to each other except that against *C. eragrostidis* where bavistin was found to be ineffective even at the highest tested concentration. When observed for phytotoxicity of the purified constituent, no necrotic damage was visible in the treated uninoculated leaves.

3.4. Control of grey blight, brown blight, leaf spot and diplodia leaf blight in tea

Treatment of tea plantlets with formulations prepared using the compound purified from *X. strumarium* leaf extracts 1 h before inoculation with the test pathogens at 2 mg ml^{-1} concentration reduced the disease incidences significantly (P < 0.05) when compared to control sets (Table 3). Severe disease development was noticed in the plants inoculated with the pathogens but not treated with *X.*

Table 3
Effect of application of bioformulations prepared from *X. strumarium* crude (4 mg ml^{-1}) and purified (2 mg ml^{-1}) leaf extract on the control of disease caused by *C. camelliae* (brown blight), *C. eragrostidis* (leaf spot), *L. theobromae* (leaf blight) and *P. theae* (grey blight) in young tea plants 16 days after inoculation with the pathogens.

Tea cultivar	Treatment	Mean foliar disease index/plant after 16 d of inoculation[a]			
		C. camelliae	*C. eragrostidis*	*L. theobromae*	*P. theae*
TV-18	Control[b]	22.5 ± 1.0	17.8 ± 0.9	11.5 ± 0.5	20.7 ± 1.2
	X. strumarium treated (purified compound)	3.1 ± 0.4	1.3 ± 0.3	2.4 ± 0.4	4.9 ± 0.7
	X. strumarium treated (crude)	6.5 ± 0.6	4.7 ± 1.0	5.2 ± 0.07	10.1 ± 1.1
	Fungicide control[c]	5.9 ± 0.5	15.6 ± 0.7	2.6 ± 0.3	4.2 ± 0.4
CD at 5% level		0.46	0.53	0.29	0.63
TV-30	Control[b]	14.6 ± 0.9	10.6 ± 1.1	12.6 ± 1.0	11.4 ± 0.8
	X. strumarium treated (purified compound)	1.4 ± 0.3	0.7 ± 0.2	1.9 ± 0.4	1.4 ± 0.3
	X. strumarium treated (crude)	3.5 ± 0.4	2.3 ± 0.7	3.8 ± 0.6	4.1 ± 1.0
	Fungicide control[c]	2.3 ± 0.6	8.1 ± 0.7	1.3 ± 0.4	2.6 ± 0.8
CD at 5% level		0.45	0.63	0.48	0.52

[a] Mean of three replications. Data after ± indicate standard error values.
[b] Control set was sprayed only with emulsifying mixture without extract.
[c] Bavistin was used as fungicide at 2 mg ml^{-1} for comparison.

strumarium leaf extract or bavistin in both cultivars after 16 days of inoculation. But in plants that were treated with the formulation from purified extract 1 h prior to pathogen inoculation, disease incidence was reduced by 93.3% in case of leaf spot (caused by *C. eragrostidis*) followed by 90.4% in case of brown blight (caused by *C. camelliae*) in TV-30 variety (Fig. 3). The plants treated with crude extract formulation (at 4 mg ml^{-1} concentration) also showed high percentage of reduction in disease incidence. However, the maximum reduction in disease occurrence was 78.3% against *Curvularia* leaf spot followed by 76% against brown blight which was less than that obtained using purified compound. The purified preparation compared favourably with the synthetic fungicide bavistin whereas the crude extract was slightly less effective.

When observed for phytotoxicity, both pure and crude extract formulation did not show any visible phytotoxicity up to 12 days of spraying. However, after 12 days, plants sprayed with the crude extract formulation developed light brown spots on leaves at places where the extract droplets remained adhered. In comparison, the pure compound formulation did not show phytotoxicity even after 16 days of spraying.

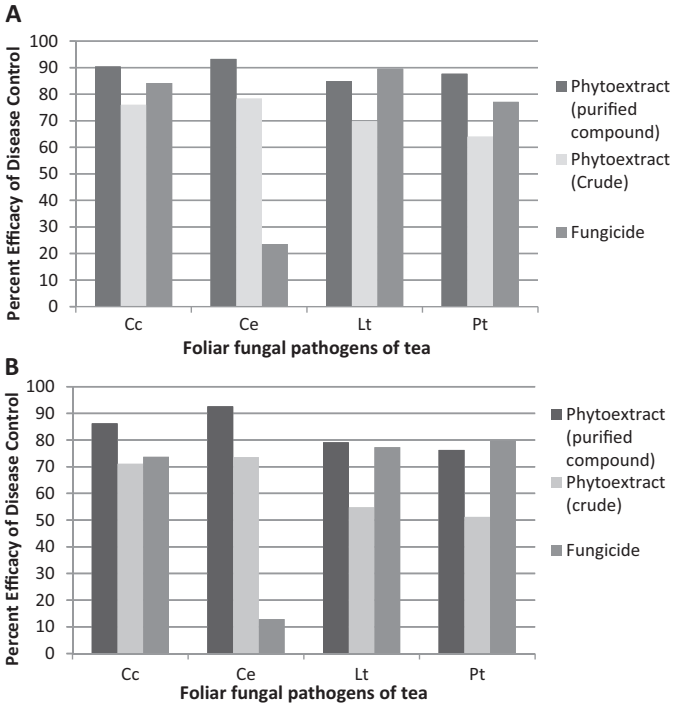

Fig. 3. Disease reduction efficacy of the crude and purified extract of *Xanthium strumarium* leaves and the fungicide bavistin against four foliar fungal diseases of tea in two clonal varieties, A: TV-18 and B: TV-30. Cc, *Colletotricum camelliae* causing brown blight; Ce, *Curvularia eragrostidis* causing leaf spot; Lt, *Lasiodiplodia theobromae* causing leaf blight and Pt, *Pestalotiopsis theae* causing grey blight.

4. Discussion

The present study shows that botanical preparation made from *X. strumarium* leaves significantly reduced the incidences of grey blight, brown blight, *Curvularia* leaf spot and *Lasiodiplodia* leaf blight in tea as effectively as fungicides. The bioactive component was isolated by bioassay guided purification methods. The purified constituent was highly effective in inhibiting the growth of a broad spectrum of plant pathogenic fungi. The importance of botanicals as fungicides in environment friendly agriculture practices has been well recognized and reports on the use of phytoextracts to combat plant pathogens are abundant (Tegegne et al., 2008; Dagostin et al., 2010; Yoon et al., 2011). The findings of this study may be significant to the tea industry as an increasing number of tea gardens are now shifting to organic farming methods.

Spectroscopic studies on the active compound purified from benzene extract of *X. strumarium* leaves revealed that this compound was a sesquiterpene lactone that was structurally similar to xanthatin. Our spectral data agreed with those obtained by other authors (Kanauchi et al., 1999; Kim et al., 2002; Ginesta-Peris et al., 1994). Little et al. (1950) was the first to report the isolation of a compound with strong antimicrobial properties from *Xanthium pennsylvanicum* which was given the name “xanthatin”. Xanthatin has been identified in solvent extracts of *X. strumarium* leaves as a potent antimicrobial substance that was antagonistic to several bacteria and yeasts and was shown to be useful in preventing contamination of food and beverages during their production (Kanauchi et al., 1999).

From our *in vitro* results it has been seen that xanthatin clearly has the potential to inhibit a number of genera of plant pathogenic fungi. Additionally other authors (Kim et al., 2002; Ginesta-Peris et al., 1994) have shown xanthatin to be effective against the plant pathogens *Phytophthora drechsleri* and *Trichothecium roseum*. The ability of xanthatin to inhibit a broad spectrum of plant pathogens is promising in the event that this compound might be considered for developing as a natural fungicide in future. In order to test the *in vivo* potential of xanthatin, it was applied against the important foliar fungal pathogens *C. camelliae*, *C. eragrostidis*, *P. theae* and *L. theobromae* on detached leaves of tea. The purified compound showed concentration dependent suppression of lesion development in tea leaves. Similar type of disease suppression was shown by *Agapanthus africanus* crude extracts against *Mycosphaerella*

pinodes in detached pea leaves (Tegegne et al., 2008). Our results showed that the minimum concentrations that did not allow lesion development ranged from 0.1 to 1.6 mg ml^{-1} for all tested pathogens. Based on this data, the extract concentration that may be suitable for use in greenhouse experiments was determined to be 2 mg ml^{-1}.

During our study, we observed that xanthatin performed better than the standard fungicide bavistin, against *C. eragrostidis*, which showed resistance to the chemical fungicide at the highest tested concentration. It also showed favourable results in comparison to bavistin against the other tested fungi. Ginesta-Peris et al. (1994) have shown xanthatin to be effective against the plant pathogens *C. gloeosporioides* and *T. roseum* which was similar to the efficacy of the tested fungicide imazalil sulphate. The prevalence of fungicide resistance is on the rise which is a serious problem in agriculture (Chung et al., 2009; Kretschmer and Hahn, 2008). This finding may be significant to the tea growers who are faced with the problem of fungicide resistance in pathogens such that it is hampering the productivity of their plantations.

Although the purified compound was found to be very effective against the tested pathogens, but it is more feasible to use crude or partially purified extracts for large scale spraying in the field. Therefore we included the crude extract in our greenhouse experiments. Since organic solvents cannot be used for spraying on tea leaves, a formulated product was developed by emulsifying the crude and pure extracts in an emulsifying mixture comprising of soybean oil and the surfactant, Tween 80. The final concentration of crude extract was fixed at 4 mg ml^{-1} based on initial preliminary results (data not shown). Wang et al. (2004) also developed an emulsified product using *Inula viscosa* leaves which effectively reduced several different crop diseases.

All the four tested tea leaf diseases were effectively suppressed by both crude and purified extract formulations of *X. strumarium* leaves. Brown blight caused by *C. camelliae* and leaf spot caused by *C. eragrostidis* exhibited maximum disease reduction. The extracts also reduced the disease incidence of grey blight and diplodia leaf blight. Spraying of extract 1 h prior to inoculation provided maximum protection against all the tested diseases. However, disease suppression, though at a much reduced level was still observed when sprayed even at 48 h prior to inoculation (data not shown). Though the medicinal properties of *X. strumarium* are well known, it has not been exploited for use as an agricultural product. One report shows that diluted fresh sap obtained from *X. strumarium* plants was highly effective in controlling Atractylis rot caused by *P. drechsleri* in pot and field trials (Kim et al., 2002). Since this plant grows abundantly as weed (Bozsa and Oliver, 1990), its utilization for disease control purposes should be advantageous.

In the present study, phytotoxicity was not visible with the purified compound; but the crude extract was mildly phytotoxic at the tested concentration after 12 days of exposure. In the work described here, we have used high concentrations of extract in order to achieve protection for the plants against all the tested diseases. But diseases like brown blight and *Curvularia* leaf spot could be checked at much lower concentrations thereby decreasing the chances of phytotoxicity. Carboxyatractylosides found in seeds and seedlings are responsible for the toxicity of *X. strumarium* (Cole et al., 1980; Cutler and Cole, 1983) but adult leaves do not contain this compound (Scherer et al., 2009). Therefore, adult leaves are considered as safe and recommended for use for medicinal purposes (Scherer et al., 2009). Further chemical studies on the cause of this mild phytotoxicity in the adult leaves should be of interest. We are presently working on fractionating the crude extract to a partially purified form, so that the phytotoxicity factor can be eliminated.

Grey blight, brown blight and diplodia disease are listed as the most common diseases prevalent in tea plantations (Singh, 2005). *Curvularia* leaf spot causes major damage to the young tea plants in the nurseries (Dasgupta et al., 2005). At present, these diseases are treated by different fungicides including copper compounds which are hazardous for the environment and also have deleterious effect on plants (Saha et al., in press). It is therefore necessary that the chemical fungicides should be replaced by safer arsenals for combating the foliar fungal pathogens. Although there is an urgent need for bio-rational fungicides as more and more tea gardens are shifting to organic production, but, to our knowledge, there are very few reports of botanical products being effective for treating foliar tea diseases (Saha et al., 2005a). In the present study, while the purified compound can be used safely in the present form to control foliar diseases, the crude extract may be used with caution especially after conducting toxicity studies to evaluate its safety on mammals. Considering the high antifungal activity and low phytotoxicity of *X. strumarium* extract under the test conditions, the extract may be available for the control of various tea leaf diseases after appropriate field tests.

5. Conclusion

In conclusion, the application of *X. strumarium* leaf extracts in purified and crude formulations showed a high percentage of reduction in incidences of four different foliar fungal diseases of tea. In the present study, the sesquiterpene lactone, xanthatin was isolated from the leaf extracts which showed high *in vitro* antifungal activity against a broad range of phytopathogens. Therefore, it may be tested for protection of several other crops against a wide range of diseases. This study indicates that the formulations prepared from *X. strumarium* extracts are a potential alternative to synthetic fungicides that may be used by the tea plantations to control foliar diseases.

Acknowledgements

The authors wish to thank Prof. B. Basu and Dr. A. Nanda of the Department of Chemistry, University of North Bengal for their help in spectroscopic analysis. Financial assistance received from CSIR, New Delhi in the form of a research scheme (38(1132)/06/EMR-II dated 29.08.06) is gratefully acknowledged.

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