3.1. Literature review

The plant kingdom is a treasure house of potential therapeutic compounds. The use of herbal medicines for the treatment of diseases and infections is as old as mankind. In the last few decades, there has been an exponential growth in the field of herbal medicines. They are getting popularized in developing and developed countries owing to its natural origin, lesser side effects and cost-effective qualities (Khan and Ahmad, 2019). The World Health Organization supports the use of traditional medicines provided they are proven to be efficacious and safe (WHO, 2020a). Medicinal plants have been used as a source of remedy against many pathological conditions since ancient times in India. The present study aims to screen the plants short listed during ethnobotanical studies and also well known as medicinal plants, for their phytochemical constituents and antioxidant properties. The present study also focuses on establishing a relationship between phytochemical content of selected plants with their antioxidant potentiality. The review presents the report of a literature search on importance of different phytochemicals. Additionally, an overview of four medicinal plants, *C. excavata*, *M. oleifera, N. arbor-tristis* and *R. serpentina* selected for experimental studies are described here.

3.1.1. Plants as source of therapeutic compounds

Traditional medicine systems consist of large number of plants with medicinal and pharmacological importance and hence represent an invaluable reservoir of new bioactive molecules (Sharma et al., 2013). In the recent years, awareness about the importance of these compounds is increasing as they are the richest bio-resource not only of traditional therapeutic systems and folk medicines, but also of modern medicines, food supplements, nutraceuticals, pharmaceutical intermediates and chemical entities for chemical drugs. Moreover, compounds from the plants are easily available, less expensive, efficient and proven to have no side effects (Zaffer et al., 2015). In different previous studies, some plants have been selected for examination and have proven therapeutically effective for development of new drugs such as anticancer drugs (Dewick, 1996; Ali et al., 2000), antimicrobial drugs

(Phillipson and Wright, 1996; Sunthitikawinsakul et al., 2003), antihepatotoxic (Hong et al., 2015) compounds. Some organic compounds isolated from medicinal plants, which are primary or secondary metabolites, provide definite physiological action on the human body. So, to explore the therapeutic value of a plant and to test its prospect as a candidate for further research, aiming drug development, analysis of its phytochemical constituents, primary and secondary metabolites content in different parts like leaf, stem or root is the foremost requirement.

3.1.2. Phytochemicals or secondary metabolites

In addition to basic nutrients like carbohydrate, protein, fat, vitamins and minerals, analysis of plant materials shows presence of some phenolic compounds, tannin, saponin, alkaloids, flavonoids etc. These materials are known as secondary metabolites, which are synthesized by plants as intermediate or by-products of their metabolism (Zaffer et al., 2015). Studies show that secondary metabolites confer the therapeutic values to the plants. So, phytochemical research based on ethnopharmacological information may prove to be an effective approach to discover medicinal, therapeutic and anti-infective agents from plants (Chhetri et al., 2008).

Primary and secondary metabolites of plant metabolism, collectively called phytochemicals, are synthesized in or during different metabolic pathways (Fig. 3.1). Primary metabolites are basic needs of life, synthesized for energy, food or defence. Secondary metabolites occur in low quantities, have no obvious role in plant growth and development, but have significant ecological function as they serve to reduce the impact on insect and animal predation or provide protection against microbial attack. They have significant economic and medical value and have been found to be used in antiquity as folk remedies, soaps and essences. They include drugs, dyes and feed stocks for chemical industries (gum, resins and rubber) and variety of substances used to flavour food and drink. These bioactive substances include tannins, alkaloids, carbohydrates, protein, glycosides, terpenoids, steroids and flavonoids (Mann et al., 1978; Edoga et al., 2005). They are widely used in the human therapy,

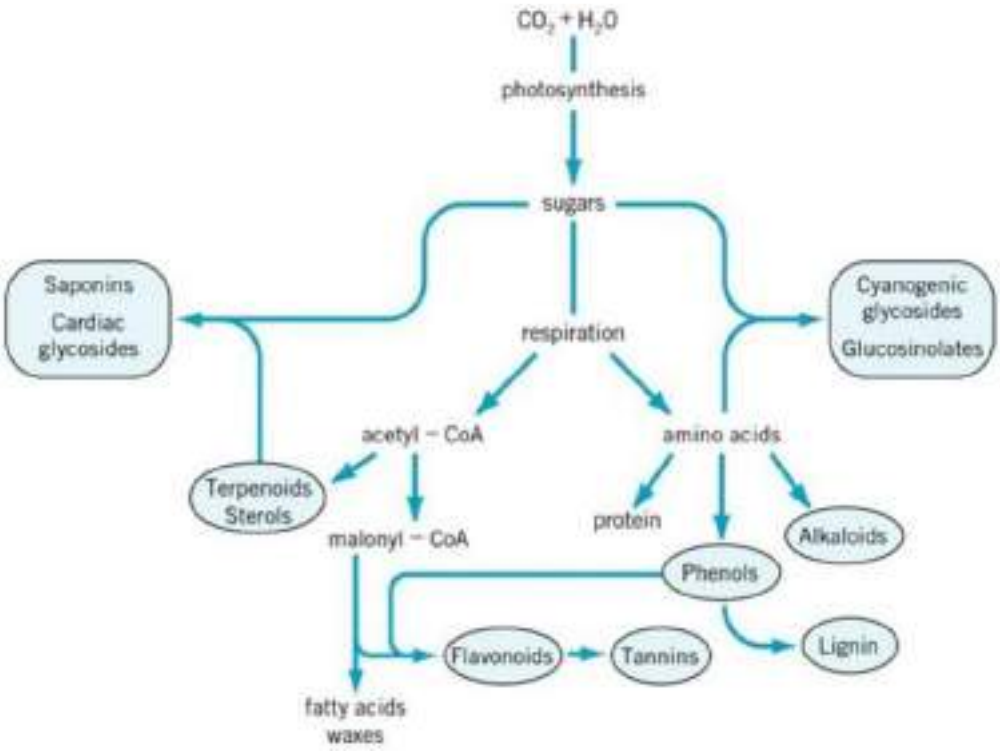

Figure: 3.1. Secondary metabolites are derived from primary metabolites

as well as in veterinary, agricultural, scientific research and many other different areas of human interest (Vasu et al., 2009). Moreover, the content of different secondary metabolites and their biological activity are shown to differ significantly with extraction procedure, solvent, time of sample collection, environment, place etc. (Shan et al., 2005).

3.1.3. Phenolic compounds

Among the secondary plant metabolites, phenolic compounds such as flavonoid, phenolic acids, tocopherols etc. are among the most ubiquitous groups (Singh et al., 2007). The phenolic compounds are of considerable interest and mostly studied in recent time. These are found to be antioxidant, antimicrobial, antiviral, anti-inflammatory, and anti-cancerous (Ignat et al., 2011). It is found that, phenolic compounds are therapeutically active on humans in various ways. More than 8000 poly phenolic compounds including 4000 flavonoids have been identified (Harborne et al., 1999).

In response to microbial infection, plants synthesize hydroxylated phenolic substances.Studies revealed that plants rich in phenolic compounds possess biological properties such as anti-apoptotic, anti-ageing, antioxidant, anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, cardiovascular protective and endothelial function improvement properties, and inhibit angiogenesis and cell proliferation activities (Han et al., 2007; Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008).

3.1.4. Flavonoids

Flavonoids have antioxidant, metal chelating potentials (Tsao and Yang, 2003). Studies show that if consumed regularly, flavonoids cause reduction in the incidence of prostate cancer (Jaganathan et al., 2014) and breast cancer (Yiannakopoulou, 2014). Isoflavones have structural similarities with ß-esterdiols, so it is referred to as 'phytoestrogen'. They are effective in prevention of atherosclerosis (Klejdus et al., 2007). Tannins interfere with protein synthesis by binding with proline rich proteins. Studies show that tannins have diverse biological forms and activities too, they work as metal ion chelator, protein precipitant and antioxidants (Hagerman, 2002).

3.1.5. Lignins, saponins and others

Lignins, produced by oxidative dimerization of phenylpropene in plant in response to pathogenic attack (Bavaresco, 2003), have potential application in cancer chemotherapy and pharmacological purposes (Saleem et al., 2005). The plant extracts containing saponins have anti-inflammatory (Just et al., 1998) and hemolytic properties (Okwu, 2004).Studies show that lycosides lower the blood pressure (Nyarko and Addy, 1990). Steroids are very important compounds, having relationship with sex hormones (Okwu, 2001). They also have antibacterial activities (Epand, 2007; Taleb-Contini et al., 2003). Alkaloids have been used for centuries for their medicinal value. They have cytotoxic (Nobori et al., 1994), analgesic, antispasmodic and antibacterial (Stray et al., 1998; Okwu and Okwu, 2004) properties.

3.1.6. Antioxidants from plants

Reactive oxygen species (ROS), super oxides like hydrogen peroxide (H_2O_2) and free radicals (hydroxyl group, $^{\cdot}OH$, O_2^{-}) are produced during normal cellular metabolism, but rapid production of such molecules can cause damage to body tissue and bio molecules, leading to cancer, asthma, diabetes, inflammatory disorder, neurodegenerative problem or premature aging (Young and Woodside, 2001). These free radicals and super oxides may biodegrade membrane lipids, cellular proteins and DNA, leading to several diseases or even death (Pourmorad et al., 2006). The lipid or fatty acid destruction causes rancidity and rotting of food items or ageing of biological tissues. Though biological systems have their own mechanisms to neutralize these naturally produced free radicals and maintain equilibrium, some external factors like environmental pollutant, radiation, chemical and physical stress may disrupt the natural procedure. So, in oxidative stress, there is an imbalance between generation and elimination of ROS and RNS (Reactive Nitrogen Species) which may cause critical diseases and aging (Serafini, 2006).

Antioxidants are the substances which delay or inhibit oxidation of oxidizable substrate by neutralizing free radical (Antolovich et al., 2002). Antioxidants work in two ways, 1. Primary antioxidants donate electron to free radical, 2. Secondary antioxidants remove ROS molecules by quenching the chain

initiating catalysts. In biological system they may exert their effects by electron donation, chelating metal ion or by regulating gene expression (Krinsky, 2002). Antioxidants may be enzymatic, like super oxide dismutase, catalase, glutathione synthase, or non-enzymatic, like, ascorbic acid, glutathione, melatonin, tocopherol, uric acid etc. Number of phenolic antioxidants like butylate hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are being used for years in food industries) to stop food rotting (food containing oil or fat).

Table: 3.1. Phytochemicals or secondary metabolites from plants used for therapeutic purposes and their mechanisms of action

Phytochemicals	Activity	Mechanism of action
Quinones	Antimicrobial	Binds to adhesins, complex with cell wall, inactivates enzymes
Flavonoids	Antimicrobial Antidiarrheal	Complexes with cell wall, binds to adhesins, inhibits release of autocoids and prostaglandins, inhibits contractions caused by spasmogens, stimulates normalization of the deranged water transport across the mucosal cells, inhibits GI release of acetylcholine
Polyphenols and Tannins	Antimicrobial Antidiarrheal Anthelmintic	Binds to adhesins, enzyme inhibition, substrate deprivation; complexes with cell wall, membrane disruption, metal ion complexation; Makes intestinal mucosa more resistant and reduces secretion; astringent action Increases supply of digestible proteins by animals by forming protein complexes in rumen; interferes with energy generation by uncoupling oxidative phosphorylation
Coumarins	Antiviral	Interaction with eucaryotic DNA
Terpenoids and essential oils	Antimicrobial Antidiarrheal	Membrane disruption Inhibits release of autocoids and prostaglandins
Lectins and Polypeptides	Antiviral	Blocks viral fusion or adsorption, forms disulphide bridges
Glycosides	Antidiarrheal	Inhibits release of autocoids and prostaglandins
Alkaloids	Antimicrobial Antidiarrheal Anthelmintic	Intercalates into cell wall and DNA of parasites inhibits release of autocoids and prostaglandins, possess anti-oxidating effects, thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on CNS causing paralysis
Saponins	Antidiarrheal Anticancer Anthelmintic	Inhibits histamine release *in vitro,* possesses membrane permeabilizing properties, to vacuolization and disintegration of teguments
Steroids	Antidiarrheal	Enhance intestinal absorption of Na+ and water.

But carcinogenic nature of some synthetic antioxidants along with high volatility and/or instability shifted the attention of both manufacturers and researchers towards the search of antioxidants of natural origin (Lourenco et al., 2019).

Plant secondary metabolites like phenolic compounds and flavonoids are found to be working as antioxidants in various studies. It has been reported that there is a reciprocal relationship between dietary intake of antioxidant rich food or plants and incidence of disease in human. Strong antioxidant properties are found in berries, cherries, citrus fruits, olives, green tea etc. Green tea contains up to 30% of its dry weight as phenolic compounds (Rio et al., 2013) and is a good antioxidant. Attempts have been made to explore antioxidant properties in regular food and vegetables like potato, spinach, legumes, tomatoes and seasonal fruits (Furuta, 2010; Wang, 2008) with positive results.

3.1.7. Overview of selected medicinal plants

3.1.7.1.*Clausena excavata*

Common Names: Agnijol, Pink lime berry, Cama, Cemama, CerekHitam, KemantaHitam, Seemere etc. *C. excavata* (Fig. 3.2) is a wild woody shrub (6 m-1m) belonging to Rutaceae family (Burkill, 1935). It is a terrestrial, tropical plant, with bushy appearance. The native distribution of the plant covers an area stretching along the Himalayas (Manosroi et al., 2005). India, Myanmar, South China, Taiwan, Philippines, Indonesia are the countries where at least fourteen species of genus *Clausena* are found growing wildly (Shier, 1983). This evergreen, flowering seed plant (angiosperm) can withstand heavy pruning and are free from pests and diseases, so they grow easily (Swarbrick, 1997).

3.1.7.1.1. Traditional use and established therapeutic properties of *C. excavata*

C. excavata is well-known from a long time for its medicinal value. Traditionally it is used in treatment of abdominal pain, as a detoxifying agent, and also in snake bites (Ridley, 1922). Tamil people use it as potherb (Arbab et

al., 2012). The root extract is used to treat ulceration of nose; decoction of root is consumed for bowel complaints for years. Decoction of leaves is also given after child birth (Wiart et al., 2004; Arbab et al., 2012). It is found from previous studies that the leaves of *C. excavata* are also used for cold and even in malaria, and root in powdered form is applied for decayed teeth whereas stem is consumed by indigenous people in colic with or without diarrhoea (Arbab et al., 2012). Reports of such a diversified use of *C. excavata* has been motivating traditional scientists and researchers to screen extracts of leaf, root and different parts of the plant for their biological activity. Table 3.2 shows some of such biological studies. In addition, table 3.3 shows in detail the therapeutic activities of various phytochemicals, secondary metabolites and essential oil, isolated from *C. excavata.*

Table: 3.2. Biological activity of the crude extracts of different parts of *C. excavata*

Plant Part	Extract	Biological Activity	Reference
Leaf	70% ethanol	Anti-hyperglycaemic, Anti-rhinitic	Sakong et al., 2011
Stem	Methanol	Oral toxicity	Puongtip et al., 2011
Leaf	Methanol	Antioxidant	Guntupalli et al., 2012
Root	Acetone	Cytotoxicity	Sharif et al., 2011
Wood	Aqueous	Immunomodulating	Manosroi et al., 2005

Table: 3.3. Phytochemicals isolated from *C. excavata* and their established therapeutic activities

Phytochemical	Classof phytochemical	Plant part	Biological activity	Reference
Clausena D and B	4-Prenyl carbazole alkaloids	Stem bark	Anticoagulant	Wu and Huang,1992
Clausendin nordentatin Clausarin, dentatin-cumarin	Pyronocoumarin	Root bark	Anticancer antibacterial	Albaayit et al., 2021; Wu and Furukawa, 1982)
Clausines B, E, H, I and K	Carbazole	Stem	Inhibit platelet aggregation in rat	Wu et al.,1996
Clausenamine-A	Biscarbazoles	Stem and root bark	Cytotoxic activity	Zhang and Lin, 2000
Mukonal	Limonoid	Stem bark	Antifungal	Takemura et al., 2000
Xanthoxyletin Murrayanine	Carbazole derivatives	Leaf	Antibacterial	Sunthitikawinsakul et al., 2003
Clausenolide-1 ethyl ether	Limonoid	Rhizome; root	HIV-1 inhibitort activity	Sunthitikawinsakul et al., 2003
Sansoakamine	Carbazole alkaloid	Stem	Anti-malarial	Lastra-Gonzalez, 2005
Clausine-TY Clausine-H Clausine	Carbazole Alkaloid	Stem; Bark	Cytotoxicity	Taufiq-Yap et al., 2007
Clausine-E and 2, 7 Dihydroxy-3 formyl-1 carbazole	Carbazole alkaloid	Leaf; Stem	Anti topisomerase II	Xin et al., 2008
Clausine	Carbazole alkaloid	Stem; Bark	Anti-proliferative	Zain et al., 2009
Clausendin	Pyrano-coumarins	Root	Anti-HIV	Kongkathip et al., 2010
Xanthyletine and Clausenarin	Coumarins	Root; Bark	Cytotoxicity	Sharif et al.,2011
Excavarin	Coumarins	Leaf	Antifungal	Saha et al., 2012

3.1.7.2. *Moringa oleifera*

Common Names: Subhanjana, Sainjana, Sojna, Suragavo, Shevga, Mulaga, Raktaka, Drumstick tree, Horseradish tree, Banzoil tree etc.

M. oleifera (Fig. 3.3) is the most cultivated species of the monogeneric family Moringaceae, which is native to India, the Himalayan tract, Pakistan, Bangladesh, and Afghanistan. *M. oleifera* is a perennial, softwood fast-growing, deciduous tree; its height may be up to 10-12m and diameter of its stem may be up to 45cm. The flowers are fragrant and asexual, surrounded by five unequal, yellow/white petals. Flowering can be seen once in a year, between April and June. Fruits are brown, shaped like a capsule, hanging and are 20-45 cm in size.

M. oleifera is an extremely popular tree for its medicinal property. In Philippines, moringa leaves are cooked and fed to babies, so it is also called "mother's best friend" or malunggay.

3.1.7.2.1. Traditional use of *M. oleifera*

M. oleifera is widely used for its therapeutic and nutritional value. All parts of *M. oleifera* tree are edible and consumed by indigenous people. History says ancient kings used to consume *M. oleifera* leaves and fruit to maintain mental alertness and for good skin. In India, Maurian soldiers were reported to take its leaves to get extra energy and to get relief from stress and pain (Jahn, 1996). With extensive use as food and medication, *M. oleifera* gets the title "the miracle tree" from the common people. Even dried leaves in powdered form are used as nutrient supplement (Makkar and Becker, 1997).

3.1.7.2.2. Biological activity of *M. oleifera*

The Iron and protein content and bio–availability of moringa leaves are found to be very high. Studies show 100 gm dry leaves contain 29.6 gm of protein (twice than that of milk), 28.9 mg of iron, 1924.28 mg of calcium and 15620.6 IU of vitamin A (Wangcharoen and Gomolmanee, 2013). It contains 7 times more vitamin C than oranges, and 3 times more potassium than a banana (Gopalan et al., 1989). It has anti proliferative, antiepileptic, anti-inflammatory, anti-hypertensive, anti-oxidant, anti-diabetic, anti-bacterial and antifungal activities (Table 3.4). It can also control blood cholesterol. *Moringa* seed oil, called Ben oil, resists rancidity (Tsaknis et al., 1999). Several

Figure: 3.2. Plant *C. excavata*(Agnijol)

Figure: 3.3. Plant *M. oleifera* (Drumstick tree)

phytochemical or biomolecules have been isolated and identified in previous studies from different parts of *M. oleifera* plant (Table 3.5).

Table: 3.4. Therapeutic activities of crude extracts from different parts of the plant *M. oleifera*

Plant Part	Biological Activity and used for	Reference
Leaf	Inflammation urinary tract infection, herpes, simplex virus anti-hypertensive	Chuang et al., 2007; Fahey, 2005; Faizi and Siddiqui, 1992
Leaf and root	Pulmonary disease	Omino and Kokwaro, 1993
Root	Kidney pain, flatulence	Fuglie, 2001
Bark	Stomach disorder	Navie and Csurhes, 2010
Pods	Joint pain	Fuglie, 2001
Root bark with gum	Dental caries	Fahey, 2005
Flower	Common cold	Fuglie, 2001
Seeds	Inflammation	Chuang et al., 2007

Table: 3.5. Compounds isolated and identified from different parts of *M. oleifera*

Compound	Plant part	Reference
Kaempferol 3,7-diglycopyranosyl (ß- D-glucopyranosyl-(1-2) - (αrhamnopyranosyl-(1-6)-ß-d-glucopyranoside)	Leaf	Faizi et al., 1995; Bushra and Anwar, 2008
Amino methoxysulfinylpentasilfide	Pod	Faizi et al., 1998
4-hydroxybenzaldehyde O-(4-O-acetyl-α-rhanmopyranoside)	Pod	Faizi et al., 1998
3,3´,4,4´,5,5´,7 heptahydro3-0-(ß-D galactopyranose,D-glucopyranose)	Bark, leaf	Asem and Laitonjam, 2008
Niazidin	Leaf	Francis et al., 2004
Rhamnose; α-l-pyranose-form, glycoside	Leaf	Francis et al., 2004
(4-hydroxybenzyl) carbamic acid	Pod, leaf	Faizi at al., 1998; Tiwari et al., 2011
(4-hydroxybenzyl) thiocarbamic acid	Fruit	Francis et al., 2004
p-Salcylic acid	Leaf	Strohl and Seikel,1965
4-Hydroxybenzyl glucosinolate	Leaf	Fahey et al., 2001

3.1.7.3. *Nyctanthes arbor-tristis*

Common Names: Seuli, Sephalika, Parijat, Harsinghar, Manjatpu Pavelam, Night Jasmine, Jayaparvati etc.

N. arbor-tristis (Fig: 3.4) is a large shrub of height up to 10m with rough leaves and flaky grey bark. Flowers are fragmented white with orange coloured corolla producing cluster of 2-8 together, which opens at dusk and closes at dawn (Das et al., 2008). Flowering usually occurs from July to October. It is a very well-known plant in India and is native to southern Asia, from northern Pakistan to Nepal, Northern India to Southeast Thailand. It is a terrestrial woody perennial tree with a lifespan of 5-20 years.

3.1.7.3.1. Traditional uses and biological activities of *N. arbor-tristis*

The plant is traditionally used for medication of diversified diseases or problems from ancient time in India. It is reported to be used to provoke menstruation, treat skin disease and scabies (Jain and Pandey, 2016).

Table: 3.6. Therapeutic activities of different parts of *N. arbor-tristis*

Plant part	Extract	Activity	Reference
leaves and fruits	Ethanolic	Anti-arthritis leaves and fruits extracts reduced TNF α, IL-1, IL-6	Rathore et al., 2007
Whole plant	Ethanolic	Antidiabetic, Subdued TBARS; antioxidant	Das et al., 2008
Leaves	Methanolic	Antibacterial, against *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Salmonella paratyphi*.	Mahida and Mohan, 2007
Leaves	Methanolic	Antileishmanial isolated compound, calceolarioside A works against visceral leishmaniasis	Poddar et al., 2008
Leaves	Methanolic	Hepatoprotective decrease the elevated levels of biochemical parameters of liver Hepatoregenerative in hepatic damage	Vishwanathan and Juvekar, 2010
Seed	Aqueous	Hepatoprotective against CCl4 induced hepatotoxicity	Lucas and Sekhar, 2000
Whole plant	Ethanolic	Immunostimulant, Enhanced total WBC count and DTH reaction	Tiwari et al., 2011

Table: 3.7. Different compounds derived from *N. arbor-tristis* with biological value

Chemicals isolated	Pharmacological activity	Parts of the plant	Reference
Arbortristoside-A Arbortristoside-B Arbortristoside-C	Antileishmania, Antihistaminic	Corolla Tubule	Sasmal et al., 2007
Nyctoside A	Not known	Seed	Sasmal et al., 2007
Carotenoid aglycone Ag-NY	Orange tubular calyx	Good membrane stabilizing agent	Siddique et al., 2006
4-hydroxy hexahydrobenzofuran-7-one Rengyolone	Flower	Antibacterial, larvicidal, Antimalarial	Tuntiwachwuttiku et al., 2003

Tribal people of India, especially in Orissa and Bihar use different parts of the plant for medication. Ayurveda, Unani, Sidho has referred *N. arbor-tristis* as having therapeutic values. The juice of its leaves is used as antidote against reptile venom, laxative digestive element and diuretics (Nadkarni, 1954). Crude extracts of different parts of the plant like leaf, bark, seed and root have been reported to have therapeutic properties (Table 3.6). In various previous studies, different bioactive compounds have been derived from *N. arbor-tristis* (Table 3.7).

3.1.7.4. *Rauvolfia serpentina*

Common Names: Sarpagandha, Chandrabhaga, Snakeroot plant, Chotachand, Chandrika, Harkaka etc.

The genus name of the plant was given in honour of a 16th century German botanist, physician, and explorer, Dr. Leonhard Rauwolf. The plant is established to have medicinal value for years. The root of *R. serpentina* has been used in India from centuries, for hypertension, as sedative and for its hypnotic properties (Gawade and Fegade 2012). *R. serpentina* is a glabrous herb or shrub and about 1-2ft long (Fig: 3.5). Leaves are arranged in whorls of 3-4, rarely opposite, ecliptic-lanceolate or acuminate or obovate acute. Soft

Figure: 3.4. Plant *N. arbor-tristis* (Night jasmine/Seuli)

Figure: 3.5. Plant *R. serpentina* (Sarpagandha)

plants are present with leaves that are light or dark green in colour. Flowers are white or finged with red; there are many-flowered cymes, corolla is salver-shaped. Fruits are pre-sized drupes, purple-black colour in ripe condition. Seeds are ovoid shape (Vaidyaratnam, 2010); root is branched and 0.5-2cm in thickness, long (8-15cm); on breaking it is circular and with centripetal lines. It is found in India, Pakistan, Sri Lanka, Burma, and Thailand. In India, it is widely distributed in sub-Himalayan area, from Punjab to Nepal, Sikkim and Bhutan.

3.1.7.4.1. Traditional uses and biological activities of *R. serpentina*

R. serpentina is being used since pre-Vedic era for the treatment of snake bite, thus the name Sarpagandha. It is also used for insect sting, hypertension, insomnia, psychological problem, epilepsy, gastro intestinal disorder, fever, wounds and also in schizophrenia (Ayurvedic Pharmacopoeia of India, Govt. of India, 2006). In ayurveda, it is well described as important therapeutic plant. It is used for its medical values in Siddha Unani system from long time. IUCN (International Union for Conservation of Nature) has marked it as endangered plant and its conservation is recommended. Different parts of this plant have been reported to have several biological activities (Table 3.8) and several bioactive compounds have been isolated from the plant parts (Table 3.9).

Table: 3.8. Biological activities of different parts of *R. serpentina*

Plant Part	Extract	Activities	References
Leaf	Methanolic	Antioxidant	Nair et al., 2012
Leaf	Aqueous	Antihypertensive	Ranjini et al., 2015
Root	Ethanolic	Antibacterial(*K. pneumoniae*,*K. aeruginosa*)	Kumari et al., 2013
Root and leaf	Methanolic	Antibacterial	Murthy and Narayanappa, 2015
Rhizome	Aqueous ethanolic or Methanolic	Hepato-protective	Gupta et al., 2010
Root	Methanolic	Antidiabetic	Azmi and Qureshi, 2013
Leaf	Methanolic	Antidiarrheal	Ezeigbo et al., 2012

Table: 3.9. Compounds purified from different parts of *R. serpentina* with their biological activities

Compound	Plant part	Reference
Reserpine	Root	Rosen and Shoolery, 1961
Yohambinine	Root	Lohse, 2002
Reserpinine	Root	Shamma and Richey, 1963;
Rescinnamidine	Root	Rosen and Shoolery, 1961
Rauwolfine; Rescinnaminol; Reserpenediol	Root	Bose, 1952, 1954;
Raumacline; Isoraumacline; 6 α-hydroxyraumacline	Whole plant	Endreß et al., 2007
Papaverine	Bark of the root	Han et al., 2010
7-Epiloganin	Dried root	Itoh et al., 2005
Indobine; Indobinine	Root	Okabe and Adachi, 1998
Ajmalicine; Ajmalicinial; N^+-Methoxycarbonyl	Stem, bark	Nasser and Court, 1984

3.2 Materials and methods

3.2.1. Plant sample collection and extraction

Leaves of all the four plants, *R. serpentina M. oleifera, N. arbor-tristis, C. excavata* were collected in adult stages, from large bushes in sub-Himalayan region of West Bengal, India in August – September 2013 when they grow abundantly at road side and adjoining forest areas. A voucher specimen of each of the plants was deposited in the herbarium of Department of Botany, Ananda Chandra College Jalpaiguri for identification.

Fresh leaves of each of the plants were thoroughly washed with distilled water thrice and dried at room temperature for 5–10 days in shade. The dried leaves of each of the plant (500 g) were ground to moderately fine powder using REMI Mixer Grinder (REMI group, India) and soxhlet extracted with methanol (1500 ml) for 15 h at 32 °C. The extract was concentrated to dryness under vacuum

in a rotary evaporator (Eyela, Japan). A sticky brown to black residue was obtained in every case in different amounts. These solid residues were considered as crude extracts of plants.

3.2.2 Phytochemical analysis

Tests were carried out on the extract and on the powdered specimens following standard procedures as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973) to identify the constituent phytochemicals.

3.2.2.1 Qualitative analysis

3.2.2.1.1 Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration.

3.2.2.1.2. Test for phlobatannins

Deposition of a red precipitate when the crude extract from each plant sample was boiled with 1% aqueous HCl was taken as evidence for the presence of phlobatannins.

3.2.2.1.3. Test for saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. Then 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

3.2.2.1.4. Test for flavonoids

Shinoda test

Crude extract of the leaf of each plant was mixed with a few fragments of magnesium ribbon and then concentrated hydrochloric acid was added to it drop by drop. After few minutes the appearance of pink-scarlet coloration was observed which indicated the presence of flavonoids.

Alkaline reagent test

Crude extract of plant leaf was mixed with 2ml of 2% solution of sodium hydroxide. An intense yellow colour was observed which disappeared on addition of few drops of diluted acid, indicating the presence of flavonoids in the extract.

3.2.2.1.5. Test for steroids and terpenoids

Libermann Burchards test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added to the solution. Formation of brown ring at the junction and green upper layer indicated the presence of phytosterols and formation of deep red color indicated the presence of triterpenoids.

3.2.2.1.6. Test for cardiac glycosides

Keller-Killani test

Five ml of each extract (dissolved in water) was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of cardenolides. Another ring may appear below the brown ring; while in the acetic acid layer, a greenish ring may form gradually throughout the thin layer.

3.2.2.1.7. Detection of alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered.

- **Mayer's Test:** Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow-colored precipitate indicated the presence of alkaloids.
- **Wagner's Test:** Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicated the presence of alkaloids.
- **Hager's Test:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

3.2.2.1.8. Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

- **Molisch's test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicated the presence of carbohydrates.
- **Benedict's test:** Filtrates were treated with Benedict's reagent (qualitative) and heated gently. Orange-red precipitate indicated the presence of reducing sugars.
- **Fehling's test:** Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3.2.2.1.9. Detection of organic acids

- **Oxalic acid test:** To the test solution few drops of 1% potassium permanganate and dilute sulphuric acid were added; disappearance of the colour showed the presence of organic acid.
- **Malic acid test:** To the test solution, 2-3 drops of 40% ferric chloride was added, appearance of yellowish color proved the presence of organic acid.

3.2.2.2. Quantitative analysis

3.2.2.2.1. Test for total phenol

Determination of total phenolic content was performed using the Folin–Ciocalteu assay, following the method of Kim et al., 2007 with some modifications, where the reducing capacity of sample was measured. The reagent containdhetero-polyphospho tungstate-molybdate. One mililitre of each extract (in different concentrations) was added to a test tube containing 5 ml of the Folin–Ciocalteu (F-C) reagent and 5 ml of sodium carbonate (Na_2CO_3) (20% in water, v/v) was added and vortexed. A reagent blank was prepared using 5 ml of F-C reagent, 1 ml of methanol and 5 ml of sodium carbonate (Na_2CO_3). The final mixture was vortexed and then incubated for 20 mins in the dark at 25°C temperature. The absorbance was measured at 760

nm using a UV- spectrophotometer (V 530; Jasco, Tokyo, Japan). A standard curve was prepared using serially double diluted solution of gallic acid (1000-15.625 µg/ml) in methanol: water (50:50, v/v). Total phenolic values are expressed in terms of gallic acid equivalents (GAE) in milligrams per gram dried leaf extract. All determinations were performed in triplicate. The standard error represents the mean of three replicas.

3.2.2.2.2. Test for flavonoids

Total flavonoid content of test plant extract was determined by aluminium chloride ($AlCl_3$) colorimetric method (Moreno et al., 2000), where $AlCl_3$ forms complex with hydroxyl group of flavonoid compounds. It had maximum absorbance at 420 nm. The value of flavonoid content is expressed as quercetin equivalent (QE) per gram of dried leaf extract. For that a standard curve was prepared using quercetin in different concentrations (10µg/ml, 50µg/ml, 100µg/ml, 200µg/ml). Then 3 ml of methanol was added to 1ml of plant extract or standard solution, taken in a test tube. Subsequently 200 µl of 10% $AlCl_3$ and 200 µl of 1M potassium acetate solution were added to it. Next 5.6 ml of distilled water was added and the mixture was kept for 30 mins at room temperature. Finally absorbance was measured at 420 nm in UV-spectrophotometer. Significant difference with P value <0.05 was measured by one-way analysis of variance (ANOVA). The standard error represents the mean of three replications.

3.2.3. Antioxidant activity

Antioxidant potential of plant leaf extract was estimated using 2, 2'-diphenyl-1-picryl Hydrazyl Radical (DPPH) free radical scavenging assay (Patel and Patel, 2011). For the experiment, 1ml of methanolic solution of test compound in different graded concentrations (stock 5mg of extract in 20ml of methanol) were taken in test tubes. To each sample, 0.5 ml of 0.2 µM DPPH-methanolic solution (1.6 mg DPPH in 20 ml of methanol) was added and the volume was made to 2ml with methanol. The control solution contained only DPPH. The mixture components of each test tube were mixed vigorously and allowed to stand for 30 minutes in dark at room temperature. The absorbance was

measured at 570 nm by spectrophotometer. The percentage inhibition of free radical activity was measured using following formula:

% inhibition = [(absorbance of control – absorbance of test sample)/ absorbance of control]×100

A scattered line graph of percentage inhibition of free radical activity was plotted against concentration of crude extracts and for each plant, concentration of 50% inhibition (IC_{50}) was obtained from the graph. The standard error represents the mean of three replicas.

3.3. Results

3.3.1. Phytochemical analysis (Qualitative)

The leaf extracts and the dried powder of leaf of *R. serpentina,M. oleifera, N. arbor-tristis* and *C. excavata* showed the presence of tannin, flavonoids, alkaloids, carbohydrate, protein and organic acids (Table 3.10). The leaf extracts of *R. serpentina* and *M. oleifera* tested negative for triterpenoids. The leaf extracts of *N. arbor-tristis* and *C. excavata* tested negative for saponin.

Table: 3.10. Phytochemical constituents (qualitative) of leaf extracts of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata*

Phytoconstituents	*R. serpentina*	*M. oleifera*	*N. arbor-tristis*	*C. excavata*
Alkaloids	++	+	++	+
Flavonoids	+	+	+	++
Tannin	+	+	++	+
Phlobatannin	++	++	-	+
Saponin	+	++	-	-
Steroid	+	++	+	-
Triterpinoid	-	-	+	+
Cardiac Glycoside	-	++	+	+
Organic acid	+	+	+	+
Carbohydrate	+	+	+	+
Protein	+	+	+	+

++ present in good amount, + present, - absent

3.3.2. Phytochemical constituents quantitative of leaf extracts

3.3.2.1. Phenolic content

Total phenolic content (TPC) of leaf extract was determined in terms of gallic acid equivalents (GAE) in milligrams per gram of dried leaf extract and the results are shown in Table 3.11. In a comparative graph, the total phenolic contents of four plants are shown by bar diagrams depicting the highest value for *N. arbor-tristis,* and lowest value for *C. excavata* (Fig 3.6).

Table: 3.11. Phenolic content of leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE)

Plant	Sample conc. mg/ml (M/V)	Absorbance At 760nm	GAE[a] conc. µg/ml	GAE Conc. mg/ml (C)	TPC[b] as mg GAE/gm DLE# (C x V/M)	mg GAE/gm of DLE[c] ± SEM
RS	0.025	2.902	956.133	0.956	38.25	38.24±0.00
		2.902	956.066	0.956	38.24	
		2.902	956	0.956	38.24	
MO	0.013	1.223	396.266	0.396	31.70	31.70±0.01
		1.222	396	0.396	31.68	
		1.224	396.533	0.397	31.72	
NA	0.02	2.592	852.66	0.853	42.63	42.65±0.01
		2.590	852	0.852	42.66	
		2.594	853.33	0.853	42.67	
CE	0.01	0.700	222.1	0.222	22.21	22.22±0.01
		0.701	222.166	0.222	22.22	
		0.701	222.3	0.222	22.23	

[a]GAE=Gallic acid equivalent, [b]TPC=Total phenolic content, [c]DLE=Dried leaf extract

3.3.2.2. Flavonoid content

The value of total flavonoid content (TFC) was expressed as mg of quercetin equivalent (QE) per gm of dried leaf extract. The result is shown in Table 3.12.The highest flavonoid content was observed in *M. oleifera* and lowest content in *C. excavata* (Fig. 3.7).

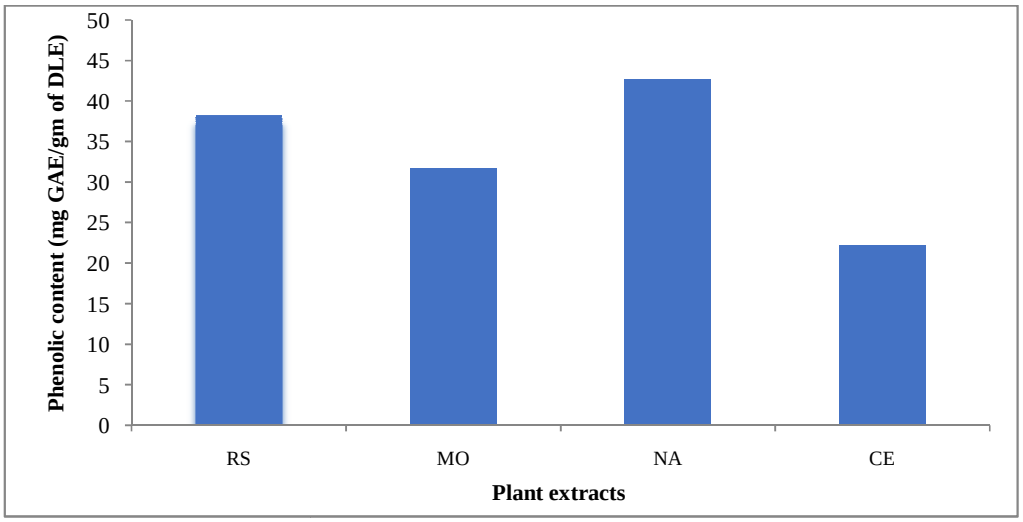

Figure: 3.6. Phenolic content of crude methanolic leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE) (GAE=Gallic acid equivalent, DLE=Dried leaf extract)

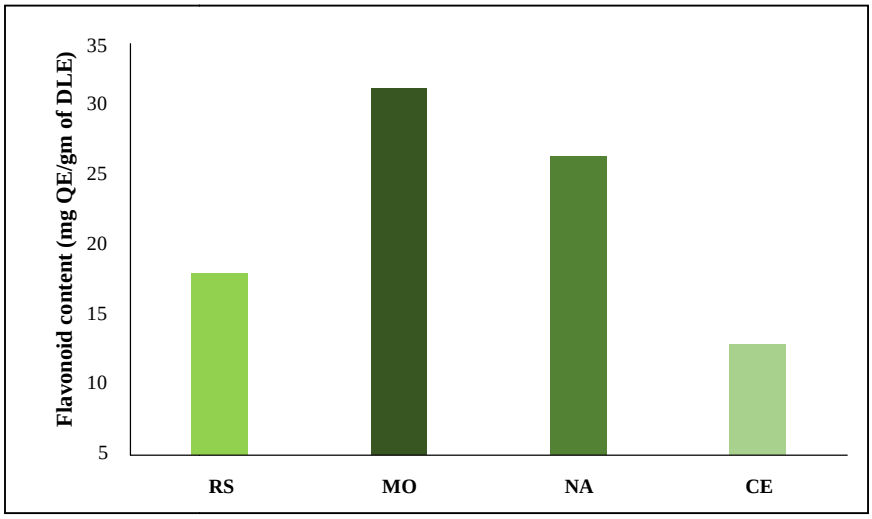

Figure: 3.7. Flavonoid content of crude methanolic leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE) (DLE = Dried leaf extract, QE = Quercetin equivalent)

Table: 3.12. Flavonoid content of leaf extracts of *R. serpentina* (RS), *M. oleifera* (MO), *N. arbor-tristis* (NA) and *C. excavata* (CE)

Plant	Sample conc. Mg/ml (M/V)	Absorbance at 420nm	QE[a] conc. µg/ml	QE[a] Conc. mg/ml (C)	TFC[b] as mg QE/gm of DLE[#] (C x V/M)	mg QE/gm of DLE[c] ± SEM
RS	0.01	1.53	156.3	0.156	15.63	15.43±0.12
		1.51	154.33	0.154	15.43	
		1.49	152.33	0.152	15.23	
MO	0.01	3.128	316.1	0.316	31.61	31.15±0.41
		3.001	303.43	0.303	30.34	
		3.117	315.3	0.315	31.5	
NA	0.01	2.58	261.3	0.261	26.13	25.4±0.68
		2.37	240.33	0.240	24.03	
		2.62	265.33	0.265	26.03	
CA	0.01	0.9	93.21	0.093	9.32	9.39±0.05
		0.915	94.823	0.095	9.48	
		0.903	93.633	0.094	9.36	

[a]QE=Quercetin equivalent, [b]TFC=Total flavonoid content, [c]DLE=Dried leaf extract,

3.3.3. Antioxidant activity

Antioxidant activities of leaf extracts of the four plants weredetermined by free radical scavenging capacity in DPPH assay. Table 3.13 shows the percentage inhibition of DPPH activity of there spective plants and IC_{50} values were also determined. All the four test samples were in concentration range of 20-100µg/ml. At the concentration of 40 µg/ml, *C. excavata* showed highest free radical scavenging activity (58%) and *R. serpentina* showed lowest activity (39%**).** Fig. 3.8 graphically shows the percentage inhibition of DPPH by all four plant leaf extracts in a given set of concentrations, representing their capacity of scavenging free radicals.

Comparison of phenolic and flavonoid content of leaf extracts of four test plants with their corresponding IC_{50} value of antioxidant (DPPH assay) activity reveals that *R. serpentina, M. oleifera* and *N. arbor-tristis* have high phenolic

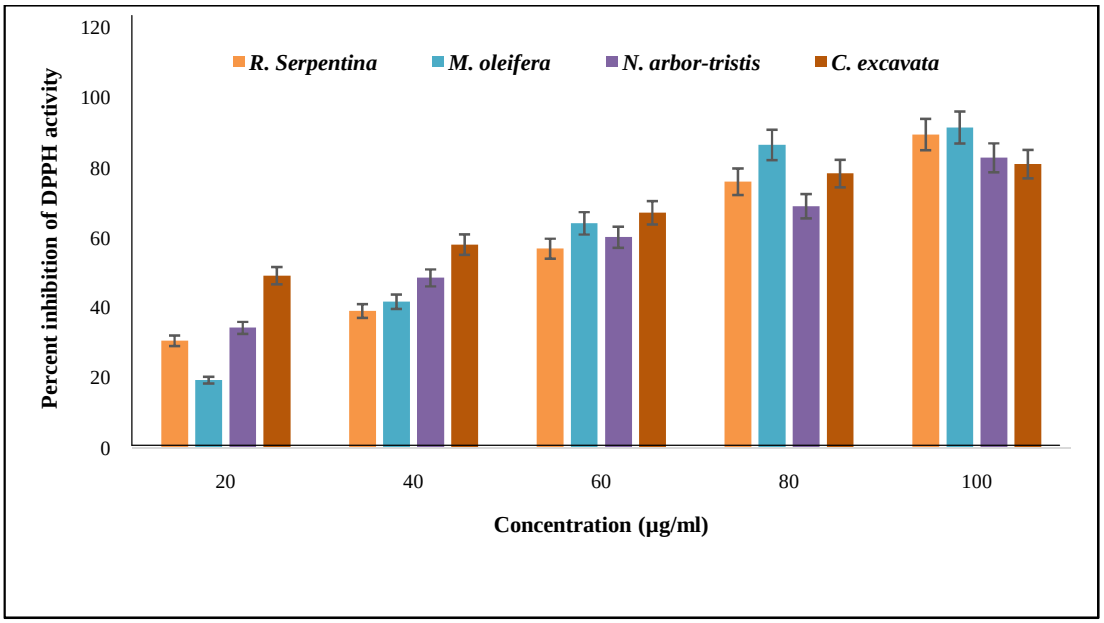

Figure: 3.8. Percent inhibition of DPPH activity of crude leaf extract of *R. serpentina*, *M. oleifera*, *N. arbor-tristis* and *C. excavata in* different concentrations.

and flavonoid content and low IC_{50} values. Only *C. excavata* with low phenolic and flavonoid content still showed lowest IC_{50}value in DPPH assay (Table 3.14).

Table: 3.13. Percent inhibition of DPPH activity by crude leaf extract of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* in different concentrations and IC_{50} values

Sample concentration µg/ml	% Inhibition			
	R. serpentina	*M. oleifera*	*N. arbor-tristis*	*C. excavata*
20	30.6	19.35	34.27	49.21
40	39.1	41.74	48.55	58.05
60	56.9	64.14	60.15	67.14
80	76	86.54	69.03	78.36
100	89.5	91.5	82.85	81.07
IC_{50}	53.6	48.5	44	20.65

Table: 3.14. Phenolic and flavonoid content of leaf extracts of four test plants with their corresponding IC_{50} values of DPPH free radical scavenging assay*

Test plant	Phenolic content (mg of GAE/gm of DLE)	Flavonoid content (mg of QE/gm of dried extract)	IC_{50} (% inhibition) of antioxidant activity(µg/ml)
R. serpentina	38.24±0.00	15.43±0.12	53.6
M. oleifera	31.70±0.01	31.15±0.41	48.5
N. arbor-tristis	42.65±0.01	25.40±0.69	44
C. excavata	22.22±0.01	9.39±0.05	20.65

*DLE=Dried leaf extract, QE=Quercetin equivalent, GAE=Gallic acid equivalent

3.4. Discussion

In this study, four well known medicinal plants, *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* were analysed for their phytochemical contents. It was observed that leaf extracts of each of the four plants gave positive result in qualitative test for alkaloid, flavonoid, terpenoid and tannin. Steroid was found to be present in three of them except *C. excavata;* saponin was found to be present in *R. serpentina, M. oleifera*; cardiac glycoside was found to be present in *M. oleifera, N. arbor-tristis* and *C. excavata;* macronutrients like protein and carbohydrate were found to be present in every plant leaf extract. In the quantitative assay, it was seen that phenolic compound was present in each of the four plant leaf extracts, with values ranging from 22.22±0.01 to 42.65±0.01 mg GAE/gm dried leaf extract. *N. arbor-tristis* contained the highest amount and *C. excavata* contained the lowest amount. Amount of flavonoid ranged from 9.39±0.05 to 31.15±0.41 mg QE/gm dried leaf extract where *M. oleifera* contained the highest amount and again *C. excavata* contained the lowest amount. It is reported that medicinal plants have high contents of organic compounds and bioactive substances including phenolic acids, tannins, alkaloids, terpenoids, steroids and flavonoid which provide definite physiological and therapeutic action on human body (Yadav and Agarwala, 2011; Criagg and David, 2011; Edoga et al., 2005). The present study confirms the previous findings. Furthermore, flavonoids are active against a wide array of microorganisms *in vitro*, which may be due to their ability to complex with extracellular and soluble proteins, and bacterial cell wall (Marjorie, 1996). Phenolic compounds have electron donating and electron withdrawing substituent in their ring structure, and thus act as antioxidant by donating hydrogen ions to highly reactive radicals (Lapornik et al., 2005).

Several studies have described that, plants rich in phenolic compounds also have antioxidant properties (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008). In our study, all of the four plants showed positive results in free radical scavenging activities in DPPH assay. *C. excavata* showed

the lowest IC_{50} implying good antioxidant property, and *R. serpentina* showed the highest IC_{50} value. In a comparison graph, it is depicted clearly that there is a relationship between phenolic and flavonoid content and antioxidant activities of respective plants. *N. arbor-tristis* had higher phenolic content and low IC_{50} value compared to *M. oleifera,* which had lower phenolic content and comparatively more IC_{50} value in percent inhibition of DPPH.A clear correlation between secondary metabolite content, mainly phenolic, and antioxidant activity of plant extract has been reported by several previous studies (Brown and Rice-Evans, 1998; Krings and Berger, 2001; Ali et al., 2008). In this study, *R. serpentina, M. oleifera* and *N. arbor-tristis* showed a good supportive result in establishing the previous findings. In contrast, *C. excavata*, which showed lowest phenolic and flavonoid content but exhibited good antioxidant property (lowest IC_{50}), indicating that active compound of different polarity might be present in the plant. Studies also reveal that the content of different secondary metabolites and their biological activity differ significantly with extraction procedure, solvent, time of sample collection, environment, place etc. (Shan et al., 2005).

In conclusion, it can be said that the leaf of test plants, *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* have shown the presence of a variety of secondary metabolites and a good source of phenolic compounds and flavonoids. The plants have also exhibited different degrees of antioxidant activities. So, they can be considered as promising source of phytochemicals and natural antioxidants for therapeutic uses. However, detailed studies are required to establish the correlation between the presence of phenolic or flavonoid contents and antioxidant activity and the role of different phytochemicals involved in antioxidant activity before using them therapeutically.