■ CHAPTER - II

Title: Extraction and characterization of Lupeol from *Xanthoxylum budrunga*, preparation of its derivatives and antimicrobial activity of each of them

Plants provide food and other life supporting commodities and are very important for the survival of man and other organisms. They protect our environment and maintain nature. The evidence of man's dependency on plant for his survival can be demonstrated by palaeo-ethnobotanical finding from prehistoric archeological sites [1]. The wild medicinal and aromatic plants are widely distributed in the mountains and forests of the eastern India. They are valuable sources of medicine for domestic and commercial purpose [2]. Although the rural people utilize wild plants for their livelihood, the scientists have recently realized the importance of such plants in rural economy.

The present chapter deals with isolation, characterization and production of four antimicrobial derivatives of naturally occurring triterpenoids of the plant *Xanthoxylum budrunga*. *Xanthoxylum budrunga* belongs to the family Rutaceae and distributed throughtout the world although substantial occurance have been reported in Zimbabwe, eastern Africa and southern Africa. In India it occurs in the Himalayan region of northeast India [3]. The plant is very common in sub-Himalayan forests.

Medicinally and commercially *Xanthoxylum budrunga* is a very useful tree. Wood is used for bundles, walking sticks and fishing rods. It is suitable for heavy construction, heavy flooring, interior trim, vechile bodies, furniture, cabinet works, main props, ladders, sporting goods, agricultural implements, toys, musical instruments, boxes, crates. [4].

The stem bark is used to treat cough, cold, boils, pleurisy, toothache and snakebite. The prickles are applied to infected wounds. The bark is used to treat chest pain and as a poultice to heal sores.Roots are used to treat mouth ulcers, sore throat and as a tonic [5] .The prickle bearing protuberances on the bole are also used by the children as toys.

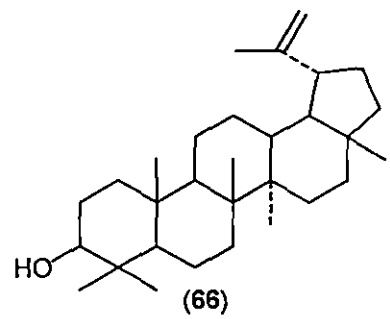
Morphologically, this plant is a dioecious, medium sized tree up to 30m tall, bole usually straight and cylindrical up to 60cm in diameter, conspicuous woody, prickled bearing protuberances, bark pale gray in younger tree to dark brown in older ones, twigs glabrous and armed with long prickles upto 5mm. Leaves alternate, imparipinnately compound with(5-7-13 phyllotaxy), leaflet up to 30cm long, stipules absent, rachis chamelled above, leaflet opposite, sessile, slightly asymmetrically oblong to lanceolate, connate and slightly notched at apex, margin finely toothed, glabrous pinnately veined with numerous lateral veins. Inflorescence terminal, panicle with flowers in clusters. Flowers unisexual, regular, small, pedicel 1-1.5cm long; sepal nearly free, 0.5mm, petals elliptical 2.5mm long; greenish yellow in color; male flower with 4 stamens; ovary rudimentary;female flowers with superior, globose ovary and short style. Fruits a globose follicle, glandular pitted, dehiscent; one seeded, black and shiny [6].

Xanthoxylum budrunga has a good medicinal as well as commercial prospect. The medicinal properties deserve more attention because of the claimed antimicrobial activities which have been confirmed by pharmacological research [7].

Section 1: Extraction and characterization of pentacyclic triterpenoids (Lupeol) from *Xanthoxylum budrunga.*

1.1 Extraction from the bark of *Xanthoxylum budrunga*: Isolation of lupeol (compound A)

Dried and powdered trunk bark of *Xanthoxylum budrunga* (2 kg) was extracted with chloroform in Soxhlet apparatus for 20 hours. Chloroform was distilled off and the gummy residue (1 kg) was taken up in ether (1.5 lt). The ether solution was washed with 10% aqueous sodium hydroxide solution (3x300 ml). The aqueous alkaline layer was thoroughly shaken with ether to remove any neutral material that might be present. The ether portion was washed with water till neutral and then dried by using Na_2SO_4. Ether was removed when a gummy residue (5g) of Lupeol was obtained. This residue dissolved in petroleum ether (30ml) was placed over a column of silica gel (30 g) developed with petroleum ether and was eluted with following solvents (table 2.1).



(66)

Lupeol

Table 2.1: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1-4	nil	-
Petroleum ether:ethylacetate(1:4)	5-8	nil	-
Petroleum ether:ethylacetate(3:2)	9-19	solid	211°-213°C

Further elution with more polar solvent did not afford any solid material

1.2. Chemical characterization of fractions:

Fractions 9-19 (Table2.1) were combined and crystallized from a mixture of chloroform and methanol. The compound was coded as 'A' and was characterized. Melting point (m.p.) was determined as 215° C. On the basis of IR, TLC and melting point the compound resembled as lupeol. Finally, the compound was confirmed as lupeol when compared with authentic sample of lupeol.

1.3: Preparation of Bromo derivatives of Lupeol:

Following steps have been carried out to prepare the bromo derivatives:

1.3.1: Hydrogenation of lupeol: Preparation of lupanol

Lupeol (coded as compound A) (5g) dissolved in a mixture of ethylacetate and acetic acid (80 ml each) was shaken in an atmosphere of hydrogen in presence of PtO_2 catalyst (0.2g) for three hours until absorption of hydrogen ceased. Ethylacetate was

removed by distillation and the solution was diluted with water whereby a white solid (4.5g) separated out which was collected by filtration. Crystalisation from a mixture of chloroform and methanol furnished colourless plates of lupanol and characterized. Melting point was determind as 204^0 C, $[\alpha]_D$ $+15^o$. On the basis of IR, TLC and melting point the compound resembled as lupanol. Finally, the compound was confirmed as lupanol when compared with authentic sample of lupanol (co-tlc, mixed m.p *etc.*).

1.3.2 Jone's oxidation of lupanol: preparation of lupanone

To a solution of lupanol (4g) in pure acetone (400 mL), Jone's reagent was added drop wise with shaking until a faint orange colour persisted. The mixture was kept at room temperature for 1 hour, dilute with water and extracted with ether. The ether layer was washed thoroughly with water and dried by using Na_2SO_4. The ether was evaporated. The residue (3.5g) dissolved in petroleum ether was chromatographed over a column of silica gel (24 g). The chromatogram was developed with petroleum ether and then eluted with the following solvents (Table-2.2).

Table 2.2: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1-4	nil	-
Petroleumether:ethylacetate(1:4)	5-8	solid	208^0-210^0C

Further elution with more polar solvent did not afford any solid material

1.3.3: Characterization of fractions:

Fractions 5-8 (Table2.2) were combined and crystallized from a mixture of chloroform and methanol. The crystallization furnished colourless needles of lupanone and characterized. Melting point (m.p.) was determined as 208^0 C. On the basis of IR, TLC and melting point the compound resembled as lupanone. Finally, the compound was confirmed as lupanone when compared with authentic sample of lupanone (co-tlc, mixed

m.p *etc.*). The characters of colourless needle shaped crystals were: m.p. 208^{0}C, $[\alpha]_D^+15$ [Lit 67 m.p. 210^0 $(\alpha)_D^+$ 16.2]

Analysis report of the compound was compared in the following table along with some characters of the isolated compound.

Analysis report	%C	%H
Present compound	84.11	11.82
Calculated for $C_{30}H_{50}O$ (authentic sample)	84.52	11.74

1.3.4.: Treatment of lupanone with N-bromo succinimide: formation of 2,2–dibromo lupanone (compound B) and 2α-bromolupanone (compound C):

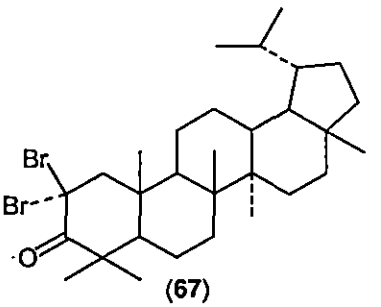
A solution of lupanone (3g) in chloroform (150ml) was mixed with dimethyl sulphoxide (75ml). N-bromosuccinimide (3.5g) was then added to it in small lots in order to keep the temperature of the reaction mixture below 25^{0}C and the mixture kept in dark for 10 days. It was extracted with chloroform and the extract washed several times with water, dried by using Na_2SO_4 and solvent removed under reduced pressure. The residue (2.8g) was chromatographed over a column of silica gel (18 g). The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 2.3).

Table 2.3: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1	oil	-
Petroleum ether	2-7	solid	208^0-209^{0}C
Petroleum ether:ethylacetate(4:1)	8-14	solid	221^0-222^{0}C

Further elution with more polar solvent did not afford any solid material

1.3.5:.Examination of fractions 2-7(Table2.3): Isolation of 2,2-dibromo lupanone (compound B) and characterization:



(67)
2,2-dibromo lupanone

The fractions 2-7 (Table2.3) showed homogeneity on TLC plate. Hence these were combined (1.1g) and crystallized from of mixture of chloroform and methanol to afford needle shaped crystals of 2,2-dibromolupanone coded as compound 'B' and characterized. Melting point (m.p.) was determined as 210^0C-211^0C and was identified as 2,2 dibromo lupanone.

Analysis report of the compound was compared in the following table along with some characters of the isolated compound.

Analysis report	%C	%H
Present compound	61.47	13.81
Calculated for $C_{30}H_{48}OBr_2$(authentic sample)	61.43	13.33

UV: λ_{MeOH} 222nm (ε = 7928)
Max 312nm (ε = 27)
Nujol
IR: ν_{max} 1722 cm-1(CO)
CD: λ_{CHCl3} 239 nm (θ = + 4590.18)
Max 320 nm (θ = - 8977.85)

1H NMR ($CDCl_3$): 0.77, 0.94, 0.97, 1.90, 1.24 (5s, 15H, 5t-CH3)
0.78, and 0.86 (2d, 6H, 2s-CH3, J = 7Hz)
3.13 and 3.64 (2d, 2H, 1-CH2, J = 16Hz) ppm

MASS: m/z at 586, 584,582 (M+), 567,569,571,
539,541,543,504,506,489,491,461,463,
426,425,409,285,283,274,231,206,205,
191,171,163,123(base peak)

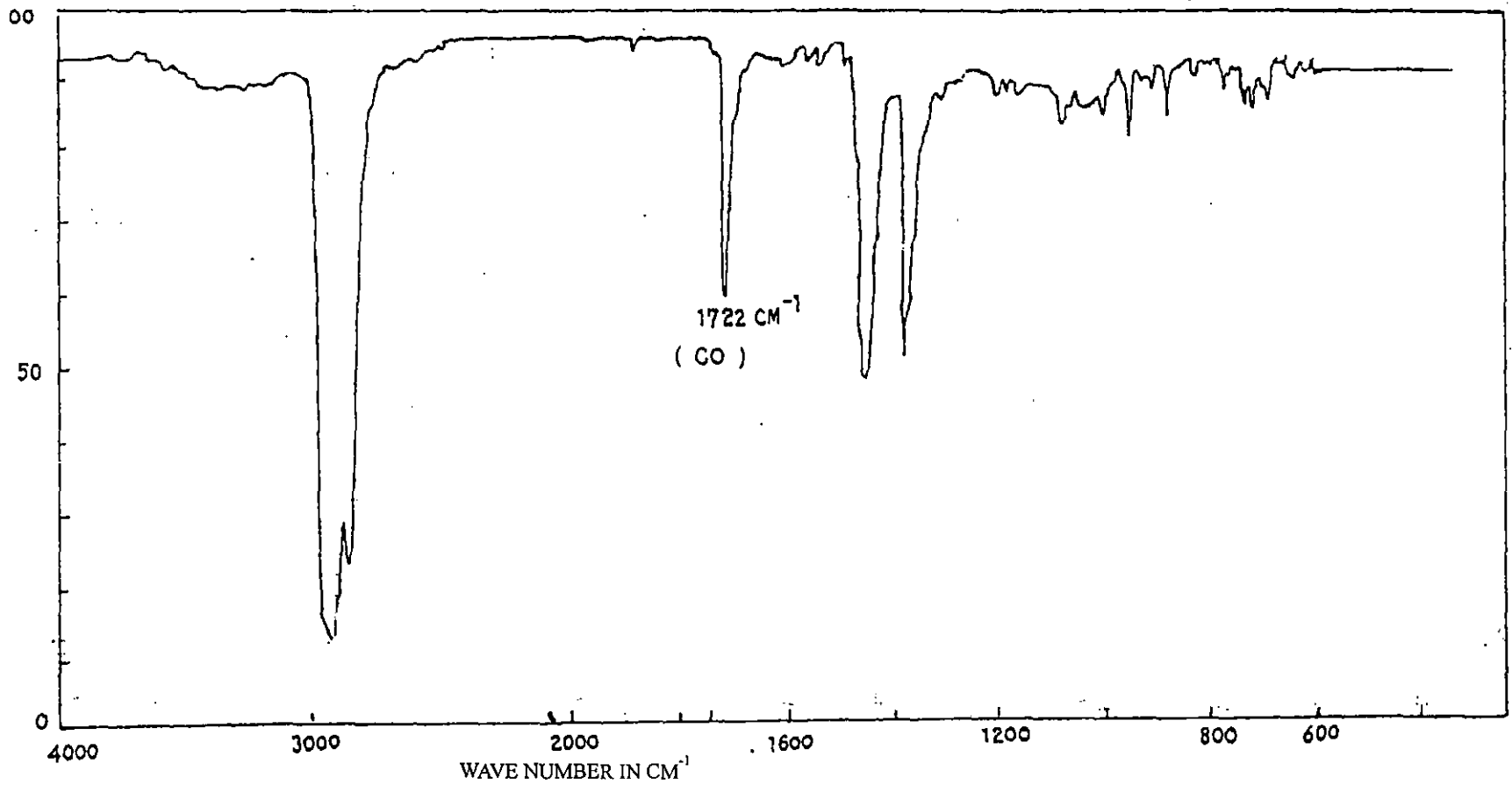


Fig. 1: IR Spectrum of 2,2-dibromolupanone (Compound B)

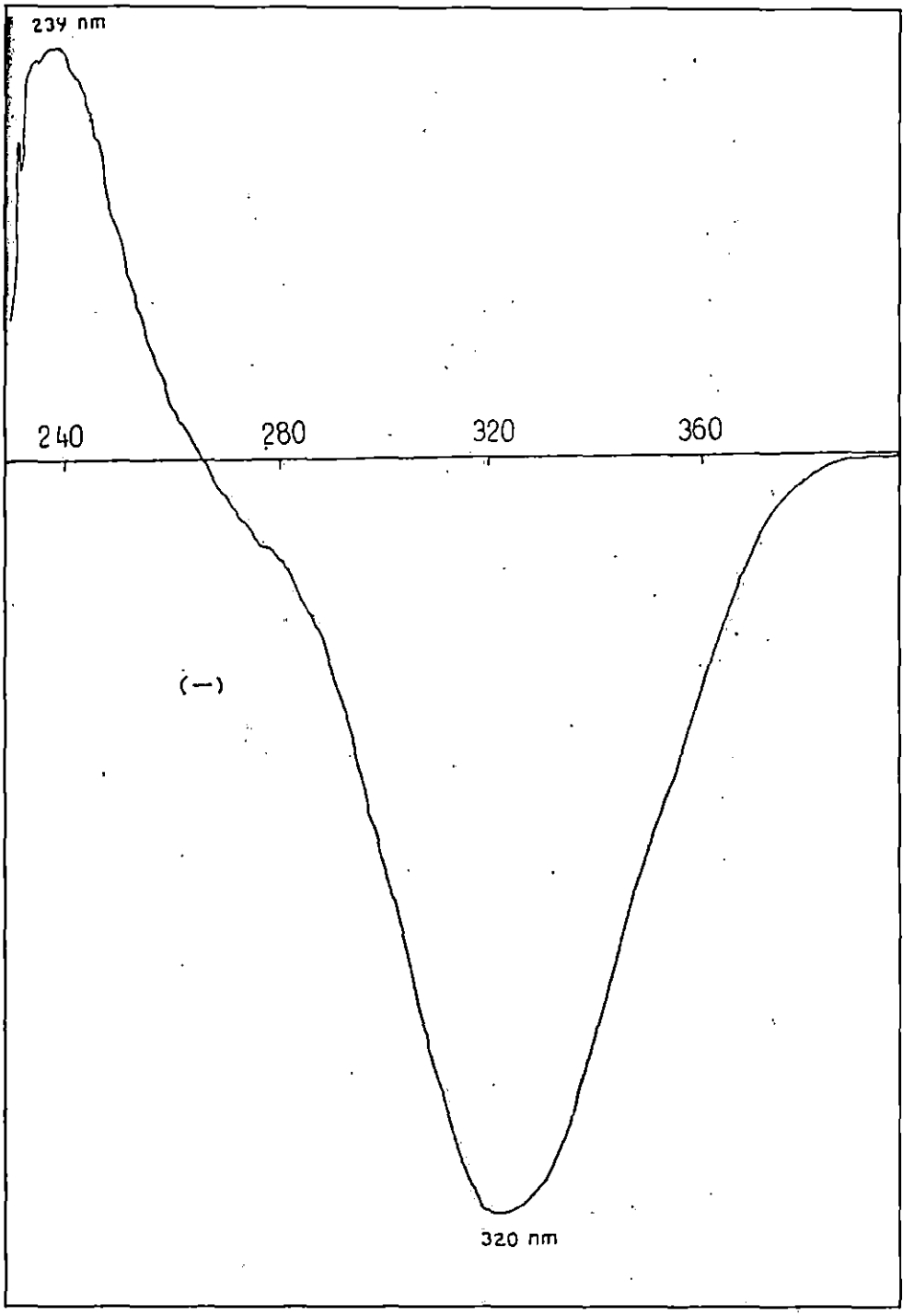


Fig. 2: CD Spectrum of 2,2-dibromolupanone (Compound B)

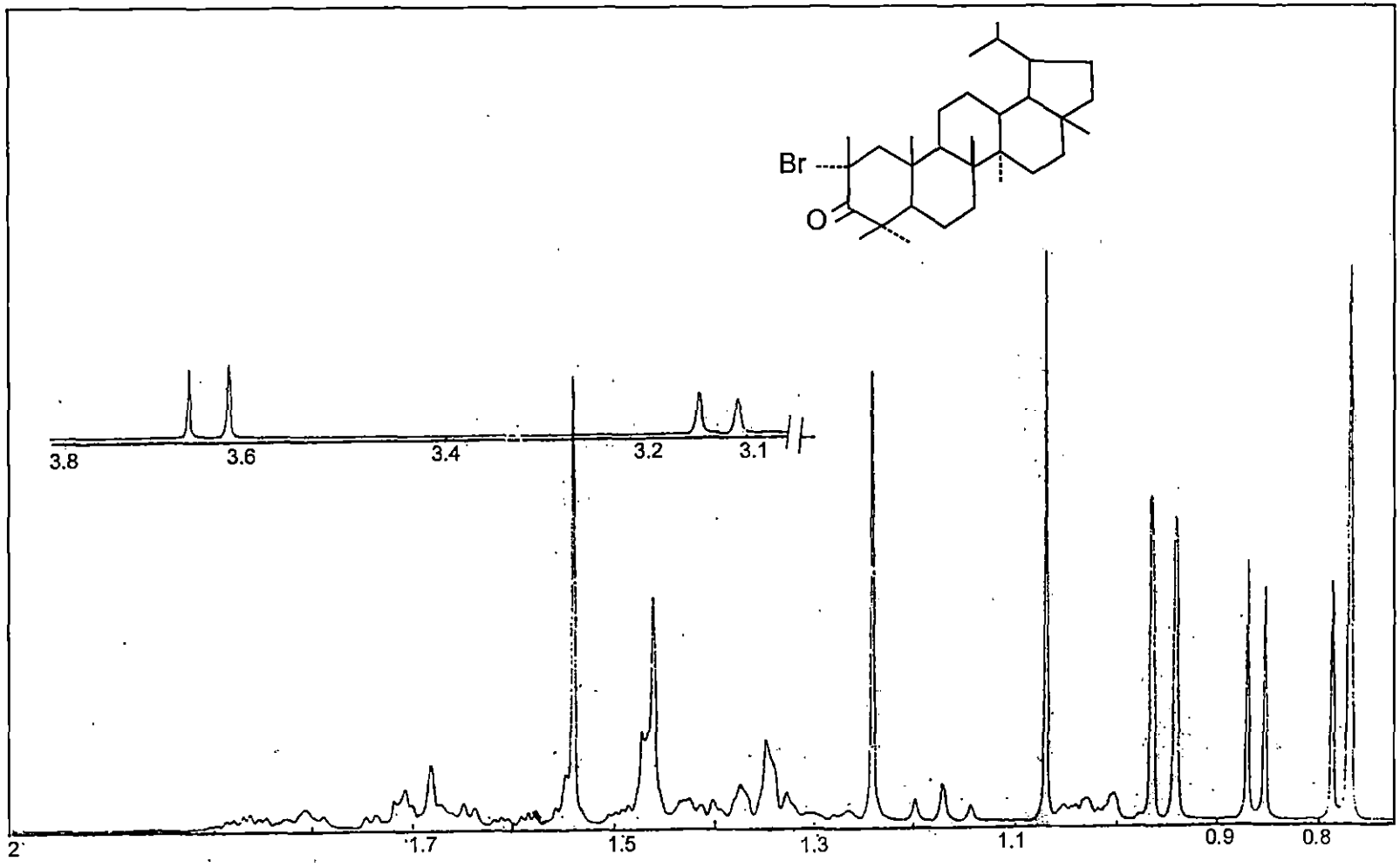


Fig. 3: NMR Spectrum of 2,2-dibromolupanone (Compound B)

$C_{30}H_{48}OB_2$

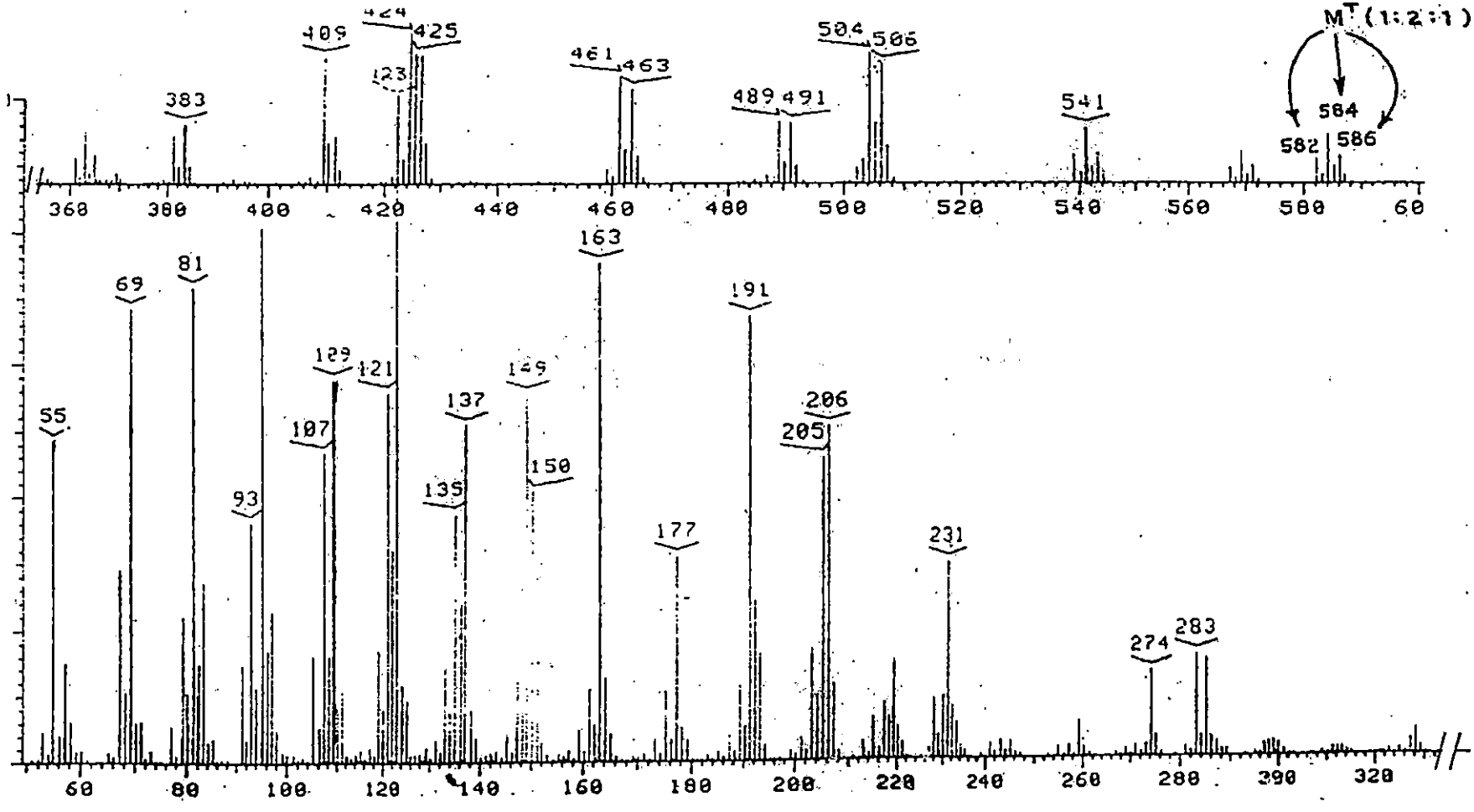
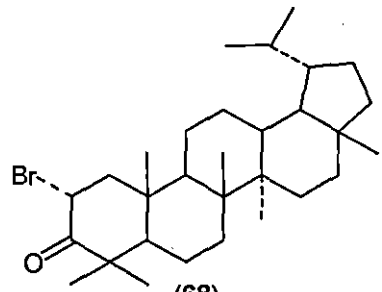


Fig. 4: Mass Spectrum of 2,2-dibromolupanone (Compound B)

1.3.6: Examination of fractions 8-14(Table2.3): Isolation of 2α-bromo lupanone (Compound C) and Characterisation:



(68)
2α-bromo lupanone

The fractions 8-14 (Table2.3) were combined (1.7g) and crystallized by chloroform –methanol mixture to afford amorphous white solid of 2α-bromolupanone coded as compound 'C'. Melting point (m.p.) was determined as 224^0C-225^0C and was identified as 2α-bromolupanone. It showed positive Belistein test for bromine.

Analysis report of the compound was compared in the following table along with some characters of the isolated compound.

Analysis report	%C	%H
Present compound	71.20	9.31
Calculated for $C_{30}H_{49}OBr$ (authentic sample)	71.15	9.68

UV: λ_{MeOH} 225 nm (ε = 7010)
Max 310 nm (ε = 42)

Nujol
IR: ν_{max} 1720 cm-1(CO)
CD: λ_{MeOH} 295 nm (θ = + 2620.82)
Max

1H NMR ($CDCl_3$): 0.77 (S, 6H, 2—CH3), 0.92, 1.10, 1.13, 1.2,
(4S, 12H, 4t—CH3)
0.76 and 0.85 (2d, 6H-2s-CH3, J = 7 Hz)
2.65 (dd, 1H, 1-c-He, J = 12 Hz and 6Hz)
2.67 (t, 1H, 1C-Ha, J =12Hz)
5.06 (dd, 1H, 2-CH, J =12 And 6Hz) ppm

MASS: m/z at 506, 504 (M+), 491,489,463,461,426,425,285, 283, 274, 206, 191, 163, 149, 123 (base peak)

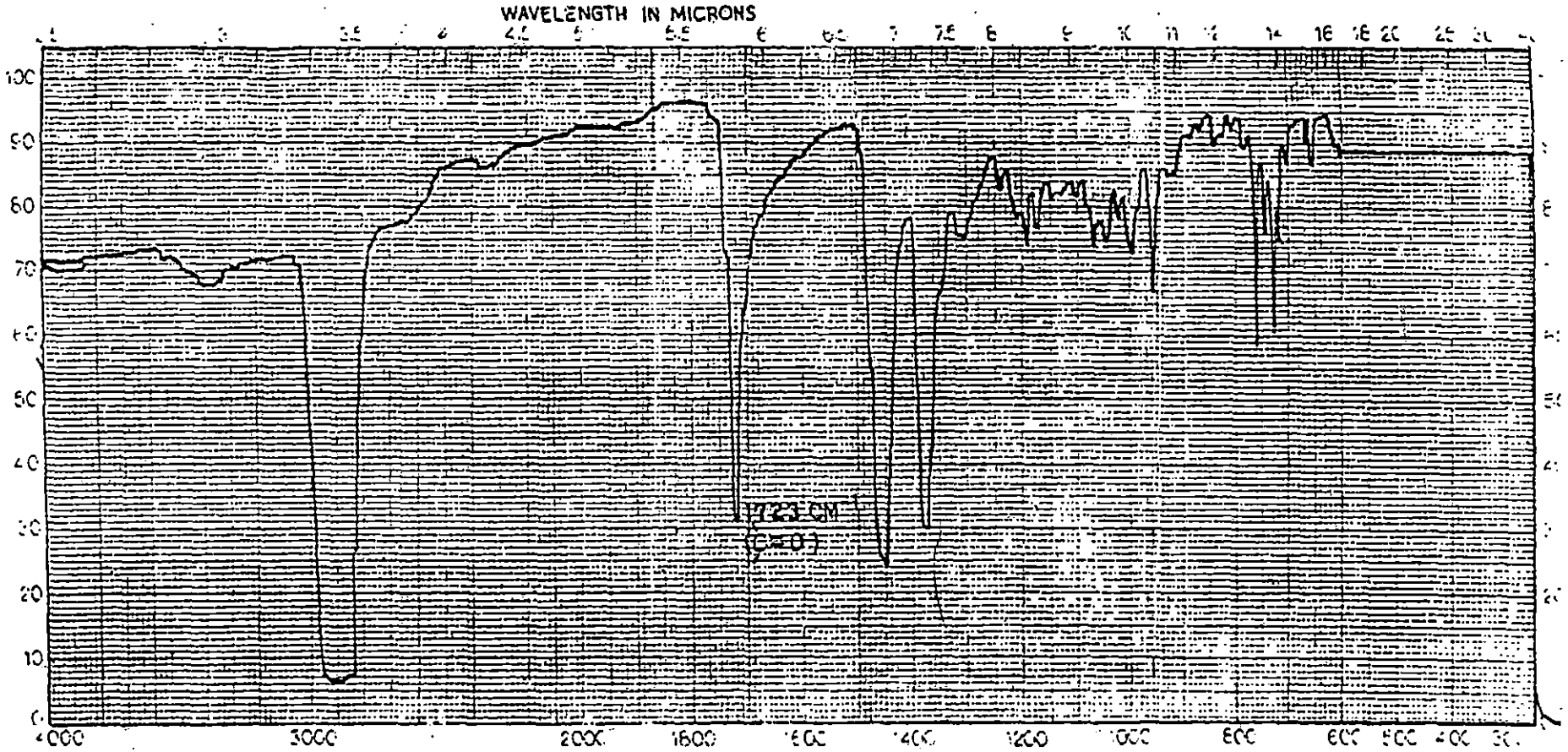


Fig. 5: IR Spectrum of 2α-bromolupanone (Compound C)

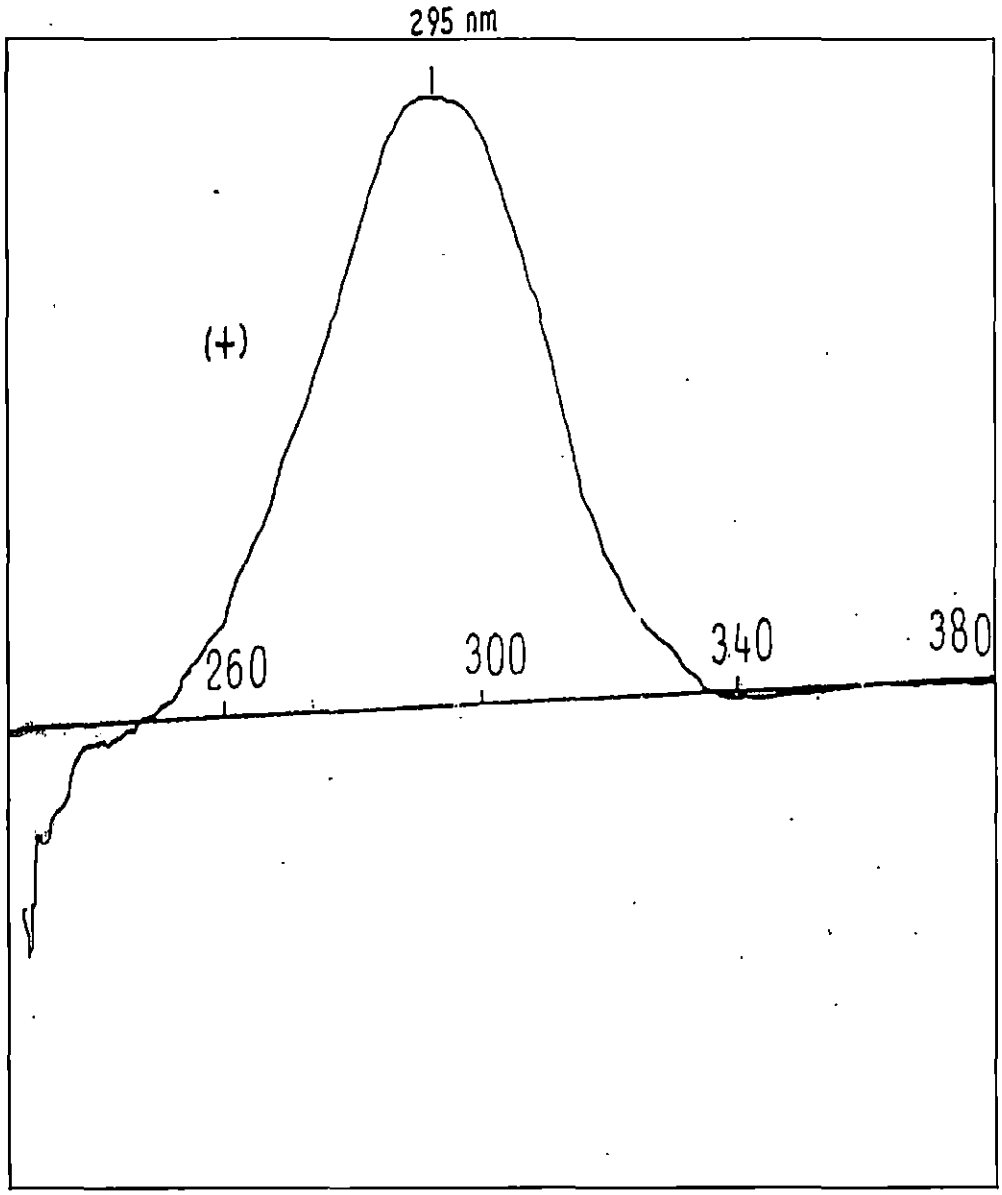


Fig. 6: CD Spectrum of 2α–ˈ bromolupanone (Compound C)

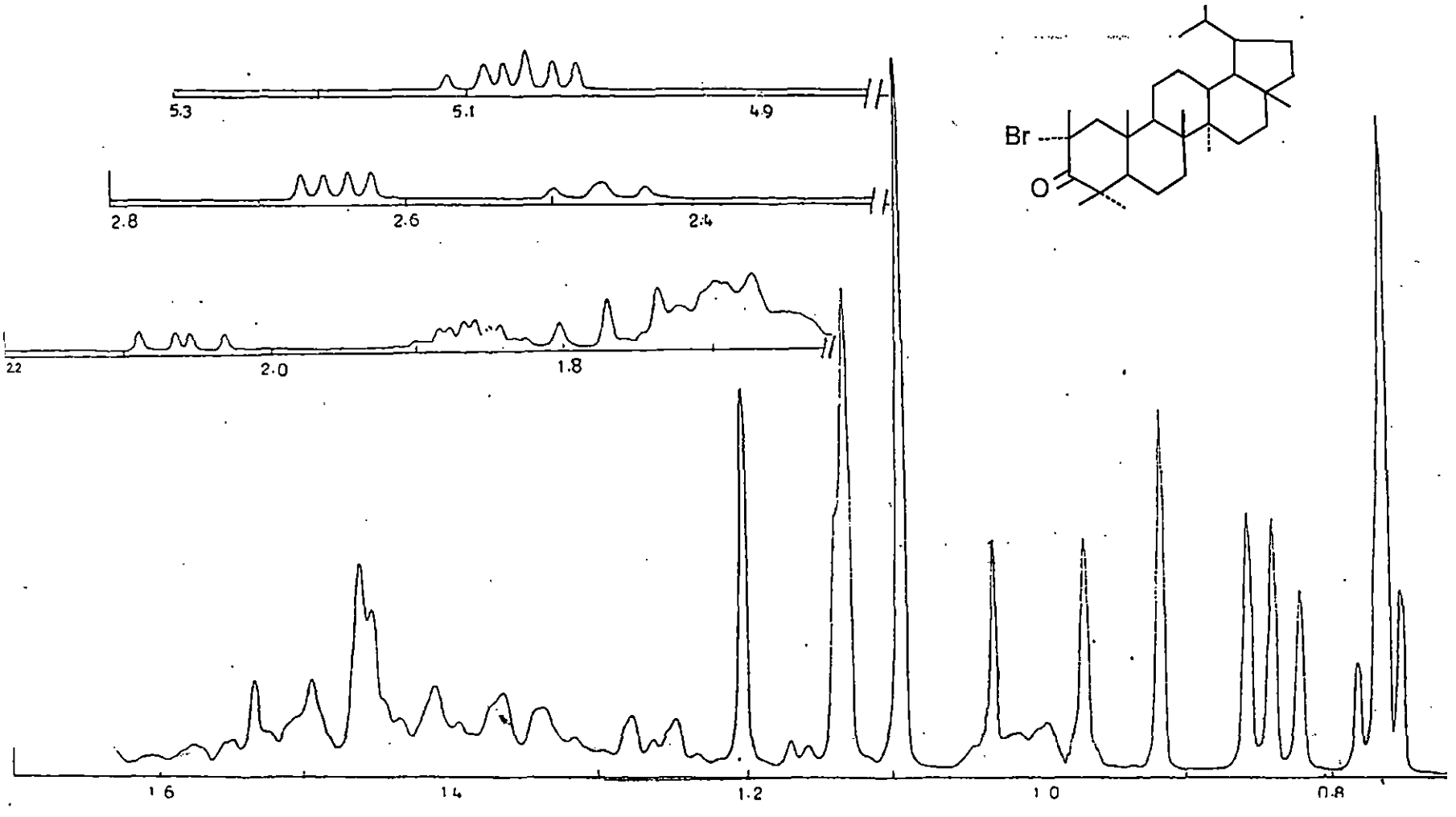


Fig. 7: ^{1}H NMR Spectrum of 2α-bromolupanone (Compound C)

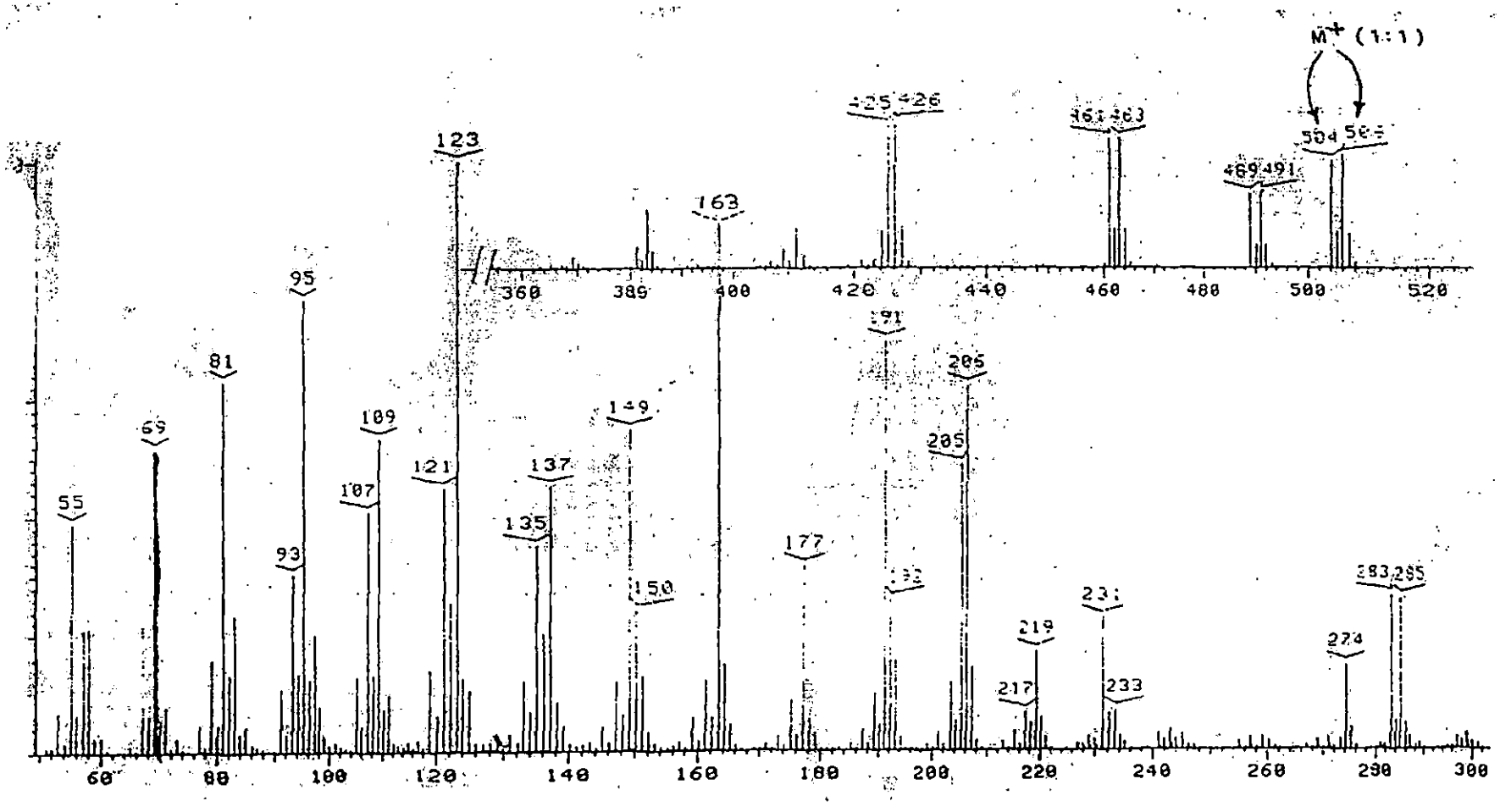
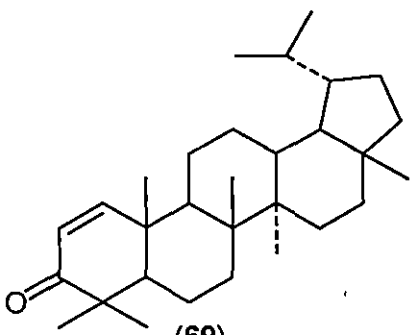


Fig. 8: Mass Spectrum of 2α-bromolupanone (Compound C)

1.3.7. Dehydro bromination of 2α-bromo lupanone with lithium bromide-N,N-dimethyl formamide: Isolation of lup-1(2)-en-3-one (compound D)



(69)
Lup-1(2)-en-3-one

Compound C (200g) (2α-bromo lupanone) was refluxed with distilled DMF (30 mL) and lithium bromide (300g) for six hours. The solid residue (180 mg) obtained after usual work up was chromatographed over a column of silica gel (10 g). The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 2.4)

Table 2.4: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1-3	nil	-
Petroleumether:ethylacetate(1:4)	4-12	solid	193^0-196^0C

Further elution with more polar solvent did not afford any solid material

Fractions 4-12 (Table2.4) showed homogeneity on TLC plate were combined (0.17g) and crystallized from chloroform-methanol mixture. The crystallization furnished colourless needle shaped crystals of lup-1(2)-en-3-one coded as compound D and characterized. Melting point of compound D was 198^0-199^0C and gave negative test for halogen.

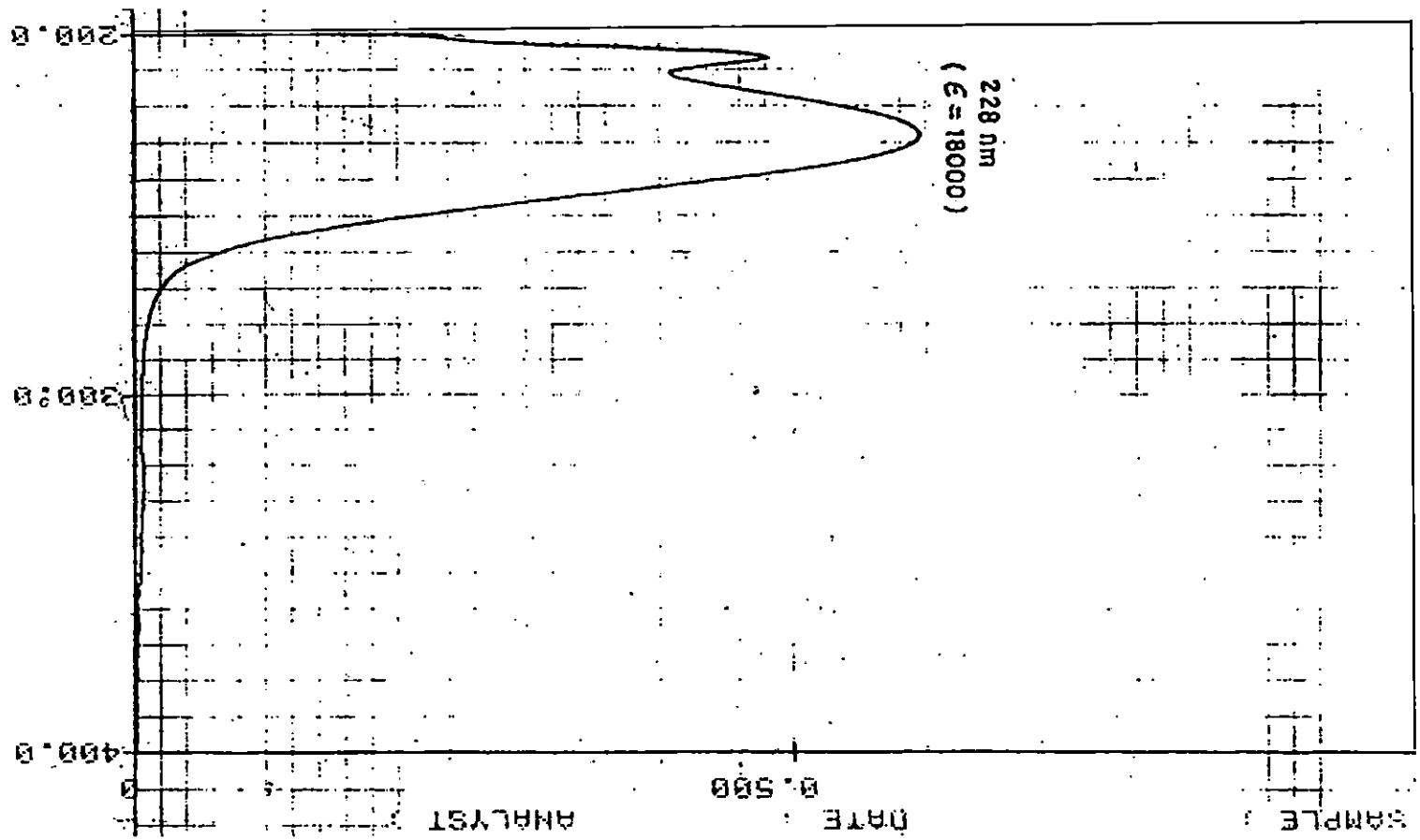


Fig. 9: UV Spectrum of 1up-1-(2)-en-3-one (Compound D)

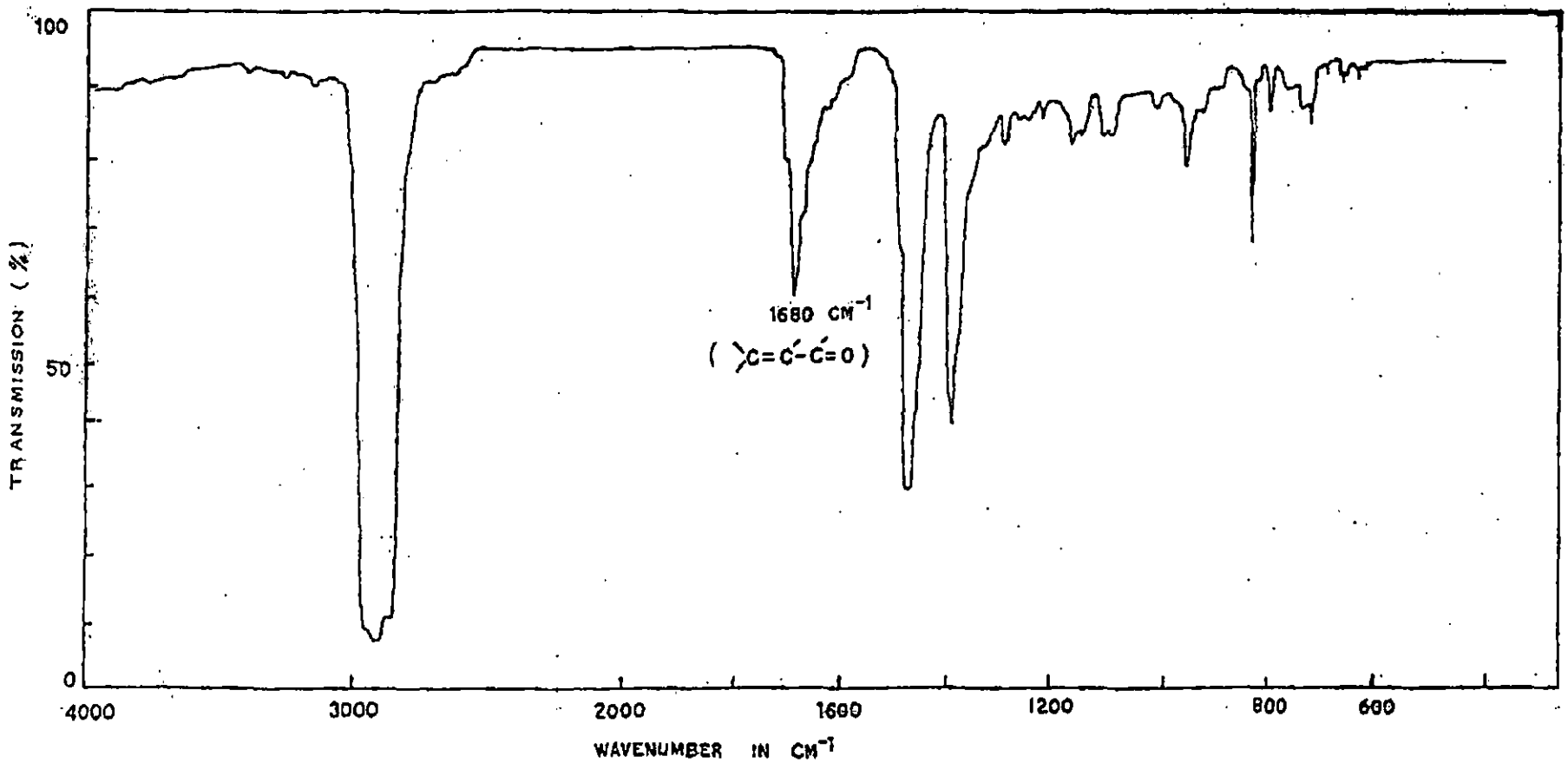


Fig. 10: IR Spectrum of 1up-1-(2)-en-3-one (Compound D)

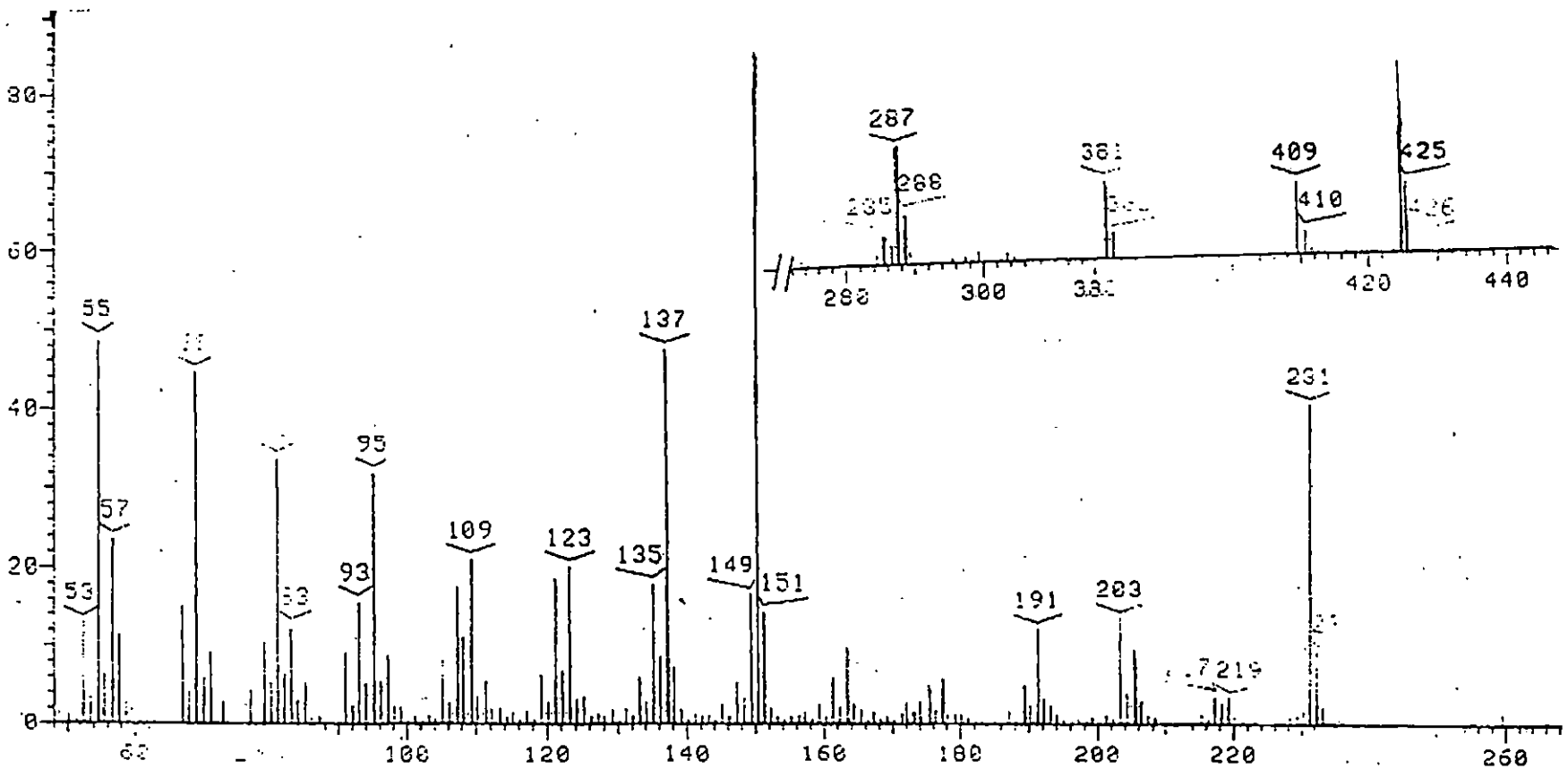


Fig. 11: Mass Spectrum of 1up-1-(2)-en-3-one (Compound D)

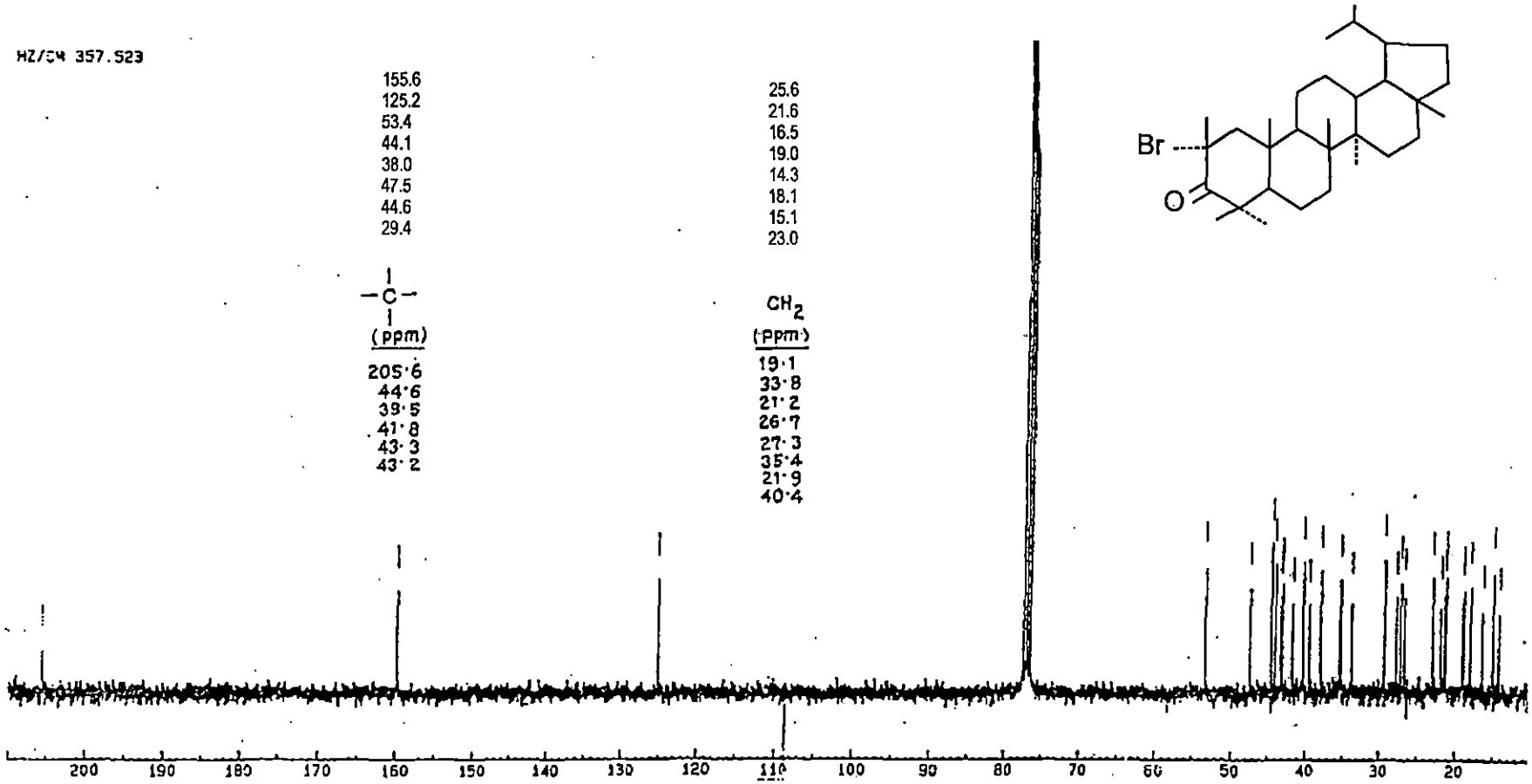


Fig. 12: ^{13}C NMR Spectrum of 1up-1-(2)-en-3-one (Compound D)

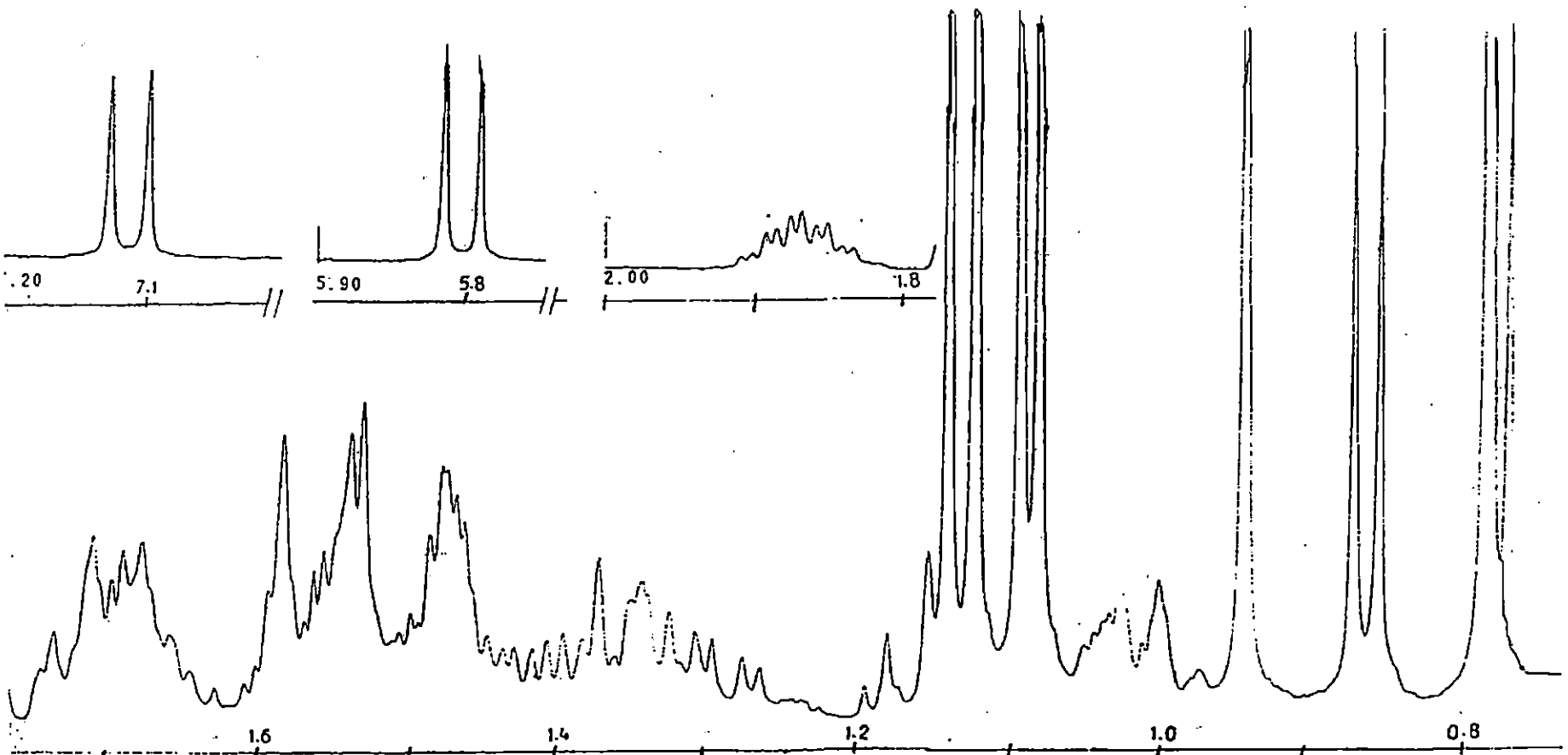


Fig. 13: ^{13}H NMR Spectrum of 1up-1-(2)-en-3-one (Compound D)

Analysis report of the compound was compared in the following table along with some characters of the isolated compound.

Analysis report	%C	%H
Present compound	84.79	11.21
Calculated for $C_{30}H_{48}O$ (authentic sample)	84.91	11.32

UV: λ_{MeOH}^{max} 228 nm (ε = 18,000)

IR: ν_{max}^{Nujol} 1680/cm (α,β-unsaturated ketone)

1H NMR ($CDCl_3$): 0.79, 0.64, 1.07, 1.08, 1.11, 1.14t (6S, 18H 6t-ch3), 0.77 and 0.86 (2d, 6H, 2S-CH_3, J = 8 Hz), 5.80 and 7.11 (2d, 2H, H-C=C-H, J = 10 Hz) ppm

MASS: m/z at 424 (M^+), 381[M--$CH(CH_3)_2$]+, 288, 287, 231, 150 (base peak), 137, 95, 69, 55.

1.3.8. Preparation of lupan [2,3-C]-1′,2′,5′-oxadiazole (coded as compound E) from lupane 2,3dioxime:

Lupan [2,3-C]-1',2',5'-oxadiazole was prepared by cyclisation of 2,3-dioximino lupane (prepared by the treatment of 2,3-diketo lupan with hydroxyl amine-hydrochloride in ethanol) in dry DMF under microwave irradiation (100W, 100^0C) for 10 minutes. The compound obtain from the reaction was purified by repeated crystallization from chloroform-methanol mixture to obtain a compound of m.p 249^0-250^0C, analyzed for $C_{30}H_{48}ON_2$, which was corroborated by mass spectral analysis that showed the molecular ion at m/z 452. The other ions appeared at m/z 437 $[M-CH_3]^+$, 409 $[M-CH(CH_3)2]^+$ (BASE PEAK) and 367, 271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81 and 55. IRspectrum of the compound showed peaks at 1620 cm^{-1} (-C=N-O)and 890 cm^{-1} for heterocyclic ring. It showed UV absorption maximum at 223 nm (ε = 5169) for disubstitiuted furazan derivative. Interestingly in the NMR spectrum different methyl group signals got separated which may be due to the presence of heterocyclic ring attached with ring. It showed the presence of six tertiary methyls of which four of them

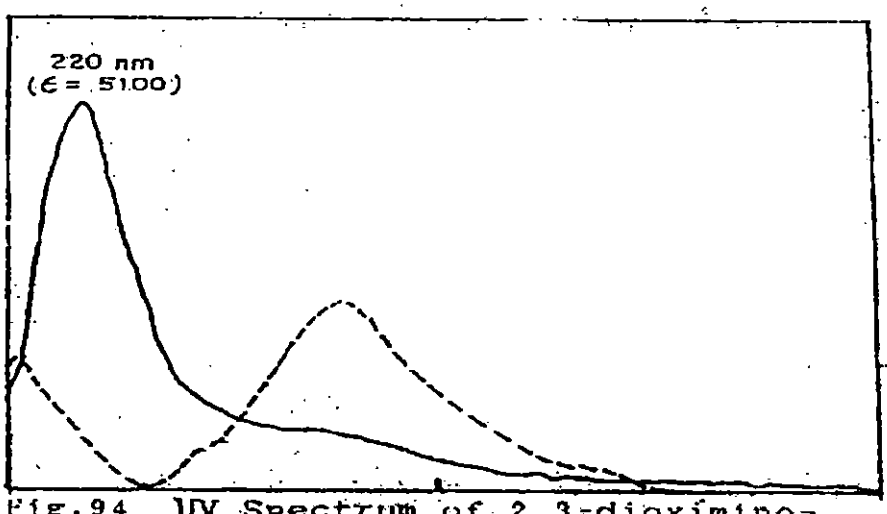


Fig.94 UV Spectrum of 2,3-dioximino-lupane(32)

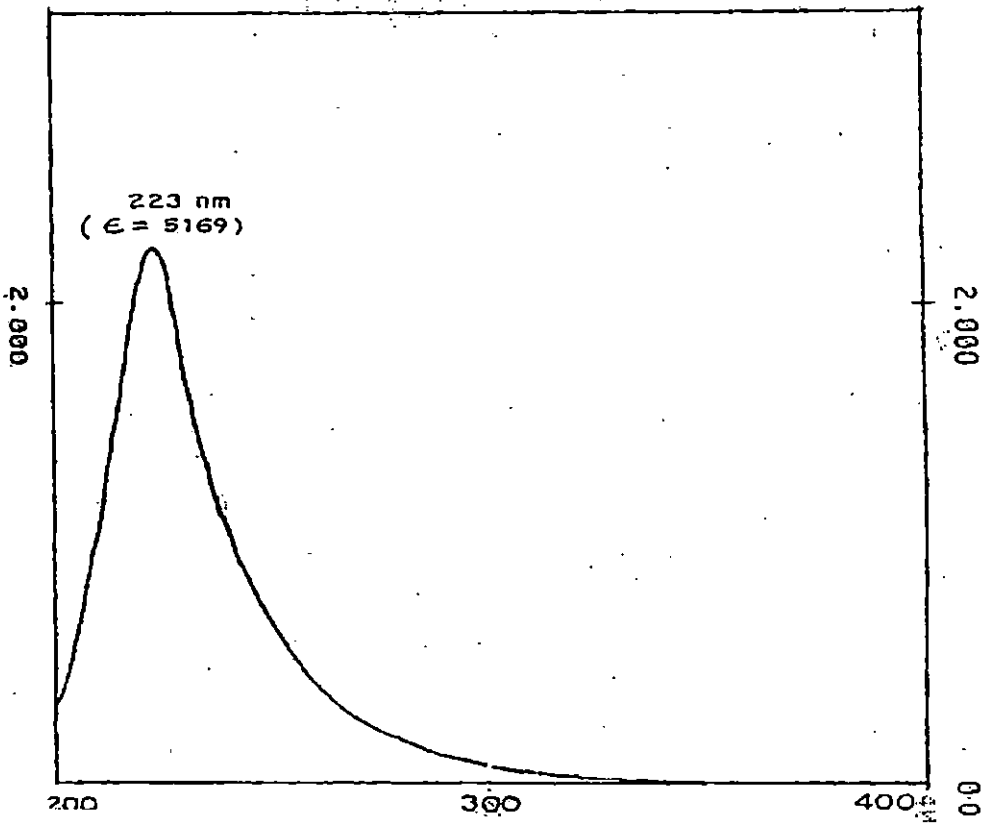


Fig. 14: UV Spectrum of lupan [2,3-C]-1′ 2′,5′-oxadiazole (Compound E)

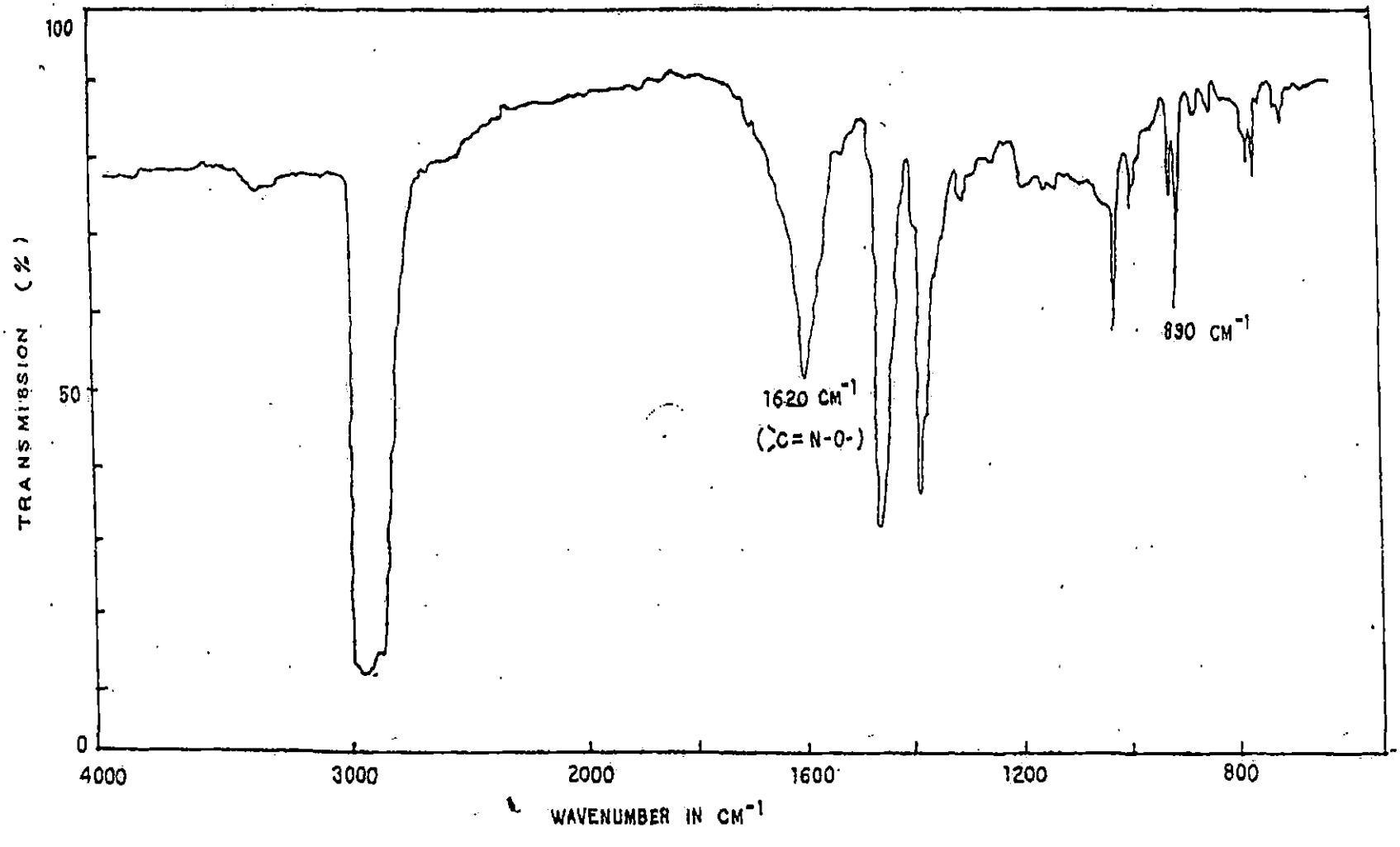


Fig. 15: IR Spectrum of lupane [2,3-C]-1′,2′,5′-oxadiazole (Compound E)

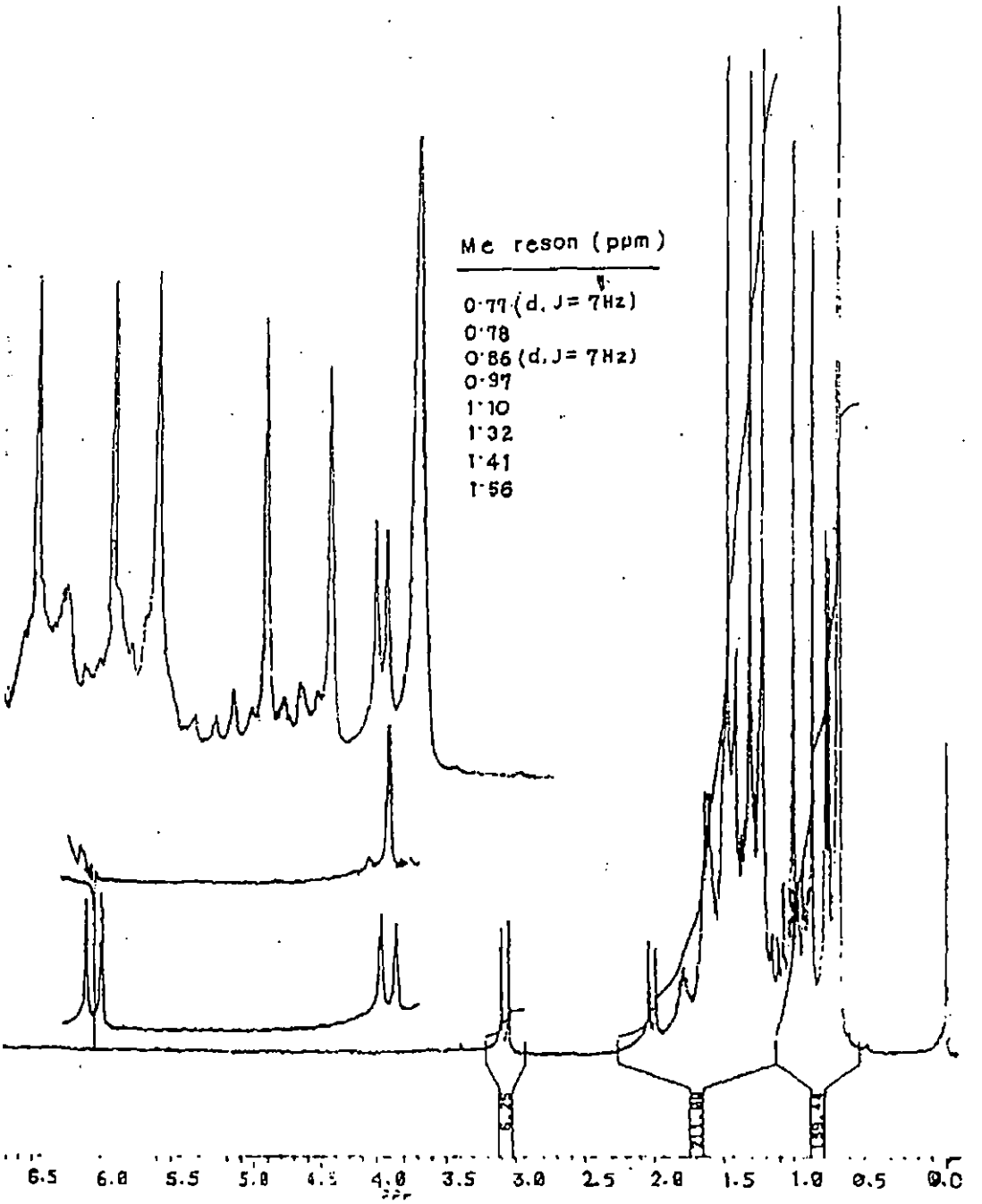


Fig. 16: ^{1}H NMR Spectrum of lupan [2,3-C]-1′,2′,5′-oxadiazole (Compound E)

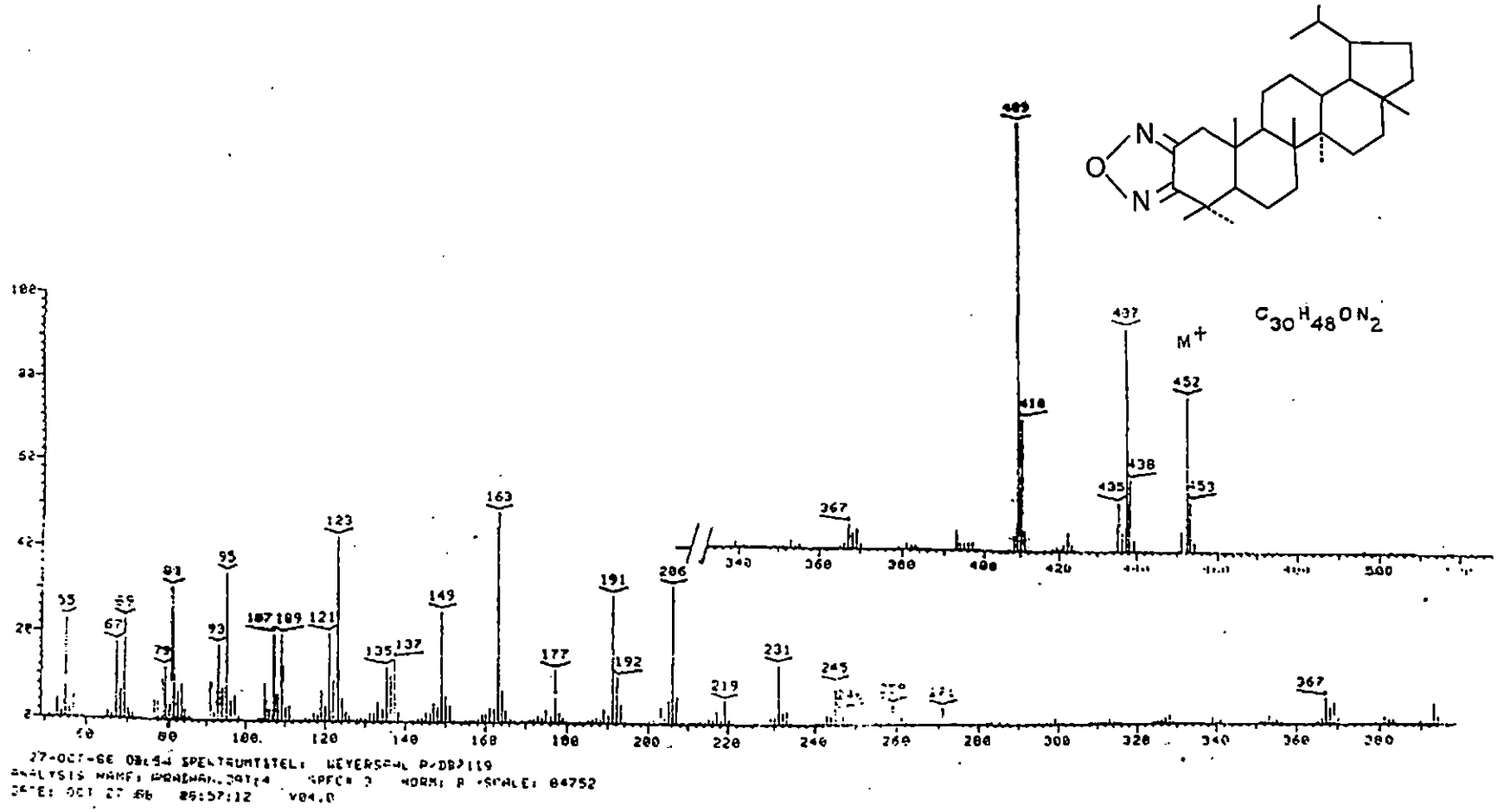
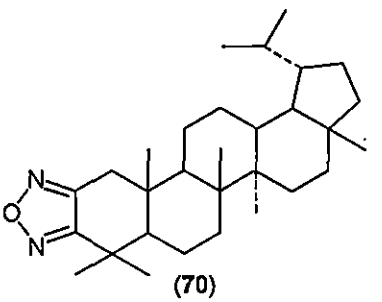


Fig. 17: Mass Spectrum of lupane [2,3-C]-1,2,5-oxadiazole (Compound E)

have been shifted downfield about 0.5 ppm in comparison to that of lupanone 1b. A pair of one proton doublets each at 2.1 (J = 16Hz) ppm and 3.15(J = 16Hz) ppm may be due to the germinal coupling of the C-1 proton which are adjacent to the furazine ring. Thus spectral analysis the structure of the compound has been established as lupan-[2,3-C]-1',2',5'-oxadiazole.



(70)

Lupane-[2,3-C]-1′,2′,5′-oxadiazole

Section 2: Antimicrobial activity

2.1: Materials and Methods

2.1.1: General Introduction: In the present study three fungal and four bacterial pathogens have been used.The details of the pathogens, their sources and their identification status have been presented in the following tables.

Table 2.5: Pathogenic fungal cultures used

Fungal culture	Source	Identified by	Identification No.
Colletotrichum gloeosporioides	Plant pathology lab (originally isolated from naturally infected brinjal leaf)	Indian Type Culture Collection, IARI New Delhi	ITCC-5446.02
Fusarium equiseti	Plant pathology lab (originally isolated from naturally infected tender brinjal stem)	Indian Type Culture Collection, IARI New Delhi	ITCC-6566.07
Curvularia eragrostidis	Plant pathology lab (originally isolated from naturally infected tender brinjal stem)	Indian Type Culture Collection, IARI New Delhi	ITCC- 4150.2K

Table 2.6: Pathogenic bacterial cultures used

Culture	Source	Identification no./ Identified by	Bacterial culture
RS	American type culture collection	Procured from American type culture collection. Identification no. ATCC-B3123	***Ralstonia solanacearum***
OB5	Plant Pathology Laboratory, Department of Botany, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr.A.Saha	***Xanthomonas* sp.**
TB3	Plant Pathology Laboratory, Department of Botany, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr.A.Saha	***Pseudomonas syringae***
PB8	Plant Pathology Laboratory, Department of Botany, University of North Bengal.	Originally isolated from naturally infected pineapple and identified* by Dr.A.Saha	***Erwinia carotovora***

RS, OB5, TB3, PB8 were used as test pathogen throughout the present study.
* Identification was done by biochemical characterization of the three bacteria.

2.1.2 Maintenance of stock cultures:

Colletotrichum gloeosporioides were grown in PDA media at 28±1°C in an incubator for 12 hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 12 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Fusarium equiseti were grown in PDA media at 28±1°C in an incubator for 48 hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 12 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Curvularia eragrostidis were grown in PDA media at 28±1°C in an incubator for 12hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 hrs. old freshly grown cultures were used. Routine subcultures were made at 45 days intervals.

Ralstonia solanacearum, a G⁻, nonsporing bacteria were grown in nutrient agar media at 37±1°C in an incubator for 24 hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Xanthomonas sp, a G⁻bacteria were grown in nutrient agar media at 37±1°C in an incubator for 24 hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Pseudomonas syringae, a rod shaped,G⁻, non spore formaing bacteria were grown in nutrient agar media at 37±1°C in an incubator for 24hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Erwinia carotovora, a G⁻, rod shaped bacteria were grown in nutrient agar media at 37±1°C in an incubator for 24hrs. The growth condition was aerobic. Finally the culture was kept in a refrigerator for storage and short time maintenance. Throughout the present study 24 hrs. old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

2.1.3 Spore germination bioassay:

Ten days old sporulated fungal culture was taken and approximately 3-5ml sterile distilled water was poured in the culture tube aseptically. Gentle scrapping was done by an inoculating needle on the agar surface. After the scrapping, the tube was shaken and

the resultant mixture was strained through cheesecloth. The filtrate was used as spore suspension. The concentration of the spores in the suspension was adjusted by adding sterile distilled water following hemocytometer count.

All the test compounds and their derivatives were subjected to bioassay against three fungal pathogens (*C. gloeosporioides, F. equiseti* and *C. eragrostidis*). The spores of the pathogens were allowed to germinate in sterile distilled water drops mounted on sterile grease free slides kept in a humid chamber in case of control. In experimental sets 30 µl of each test compound (dissolved in DMSO) was placed on the centre of a clean (grease free) microscopic slide. After that, spore suspension was mounted on the slides in the same place where the extract was applied. In solvent control set fresh solvent (DMSO) was placed and subsequently spore suspension was applied. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and the experimental slide was placed on the rods in a uniformly balanced position. Sterile distilled water was carefully poured in the petridish so that the bottom of the slide remained just above the water surface. The petridish was then covered for maintaining humid condition and finally the petridishes were incubated at 28 ± 1^0C. After 48 h of incubation, the slides were stained with lacto phenol-cotton blue and were observed under microscope. Approximately, 200 spores were observed in each slide for germination. The entire experiment was repeated thrice.

2.1.4 Bioassay by Disc diffusion Method:

Two milliliter of spore suspension was poured in a sterile petridish (90 mm diameter) and then 18 ml of the molten PDA medium was poured in the same petridish. The spore suspension and medium was mixed well and was allowed to solidify. After solidification of petridishes filter paper discs (Whatmann 40, 4mm in diameter) were dipped in different concentrations of the test compounds (100, 200, 300, 400 and 500 ppm) and then were placed on the solidified plates. In solvent control sets filter paper discs dipped in pure DMSO was placed. In distilled water control sets filter paper discs dipped in distilled water were placed on the solidified medium surface. Both experimental and control plates were incubated for period as required. Radial growth of each pathogen was measured.

In case of bacterial pathogen nutrient agar was used instead of PDA.

2.1.5. Determination of Minimum inhibitory concentration by agar cup method:

For screening of inhibitory effect of botanicals against test pathogens both spore germination bioassay technique and disc diffusion bioassay technique were followed. Minimun inhibitory concentrations (MIC) of all active components were measured following standard procedures as suggested by Portillo *et al.* 2005.[37]

A sensitive and quick petridish method as suggested by Eloff (1998)[38] was followed to determine the minimum inhibitory concentration of plant extracts against bacteria and fungi. Minimum inhibitory concentration (MIC) was determined by the micro dilution method using serially diluted test compounds. Various concentrations (Viz. 100ppm, 200ppm, 300ppm, 400ppm and 500ppm) of the plant extracts were prepared. Nutrient agar was used for the growth of bacterial strain and potato dextrose agar was used for the growth of fungi. By means of a cork borer (5 mm in diameter) a cup or well was made in a seeded (either by bacterial suspension or by fungal spore suspension) agar plate. The cup or well was filled with 50µl tested compound and was incubated in the incubators meant for bacteria or fungus as applicable. The extract diffused from the cup to a certain extent and inhibited the growth of the pathogen if it contained any antimicrobial properties. It was found that the diameter of the inhibition zone was more when the concentration of the antimicrobial properties was more. The plates containing bacteria were incubated at 37±1°C for 48 hrs. and the plates containing fungi were incubated at 28±1°C for 72hrs. The antimicrobial activity was noted on the basis of the diameter of inhibition zone where no growth was found.

2.2: Results

Compound A, chemical name 'Lupeol', extracted from the bark of *Xanthoxylum budrunga*, belongs to the family Rutaceae [details of extraction procedure have been described in chapter-II, section 1.1]. The bark was used to treat cold, cough, toothache etc. Antifungal and antimicrobial activities of the plant parts were confirmed by pharmacological research. The plant parts also had anti-inflammatory and anti-tumor effect as reported by several scientists [12-17].

From the results presented in table-2.7 it was found that the antifungal activity of lupeol was effective at higher concentrations. Out of the five concentrations tested two concentrations (400 and 500ppm) showed antifungal activity (more than 90% inhibition) in all the three fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *curvularia eragrostidis*). Percent germination of spores gradually decreased with the increasing concentration of the compound. In all the three cases 500 ppm concentration was most effective than the other concentrations.

Table 2.7: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by lupeol (compound A). (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	85	15±1.06
	200	12-40	32	68±1.50
	300	12-27	21	79±1.20
	400	08-32	05	95±1.90
	500	08-24	04	96±1.04
Fusarium equiseti	100	12-24	84	14±1.72
	200	08-20	18	82±1.38
	300	08-20	17	83±1.92
	400	04-16	10	90±1.45
	500	04-12	05	95±1.40
Curvularia eragrostidis	100	40-88	15	85±1.02
	200	32-80	11	89±1.30
	300	28-68	09	91±1.40
	400	24-56	07	93±1.55
	500	20-48	05	95±1.10

*Data after ± indicate standard error value

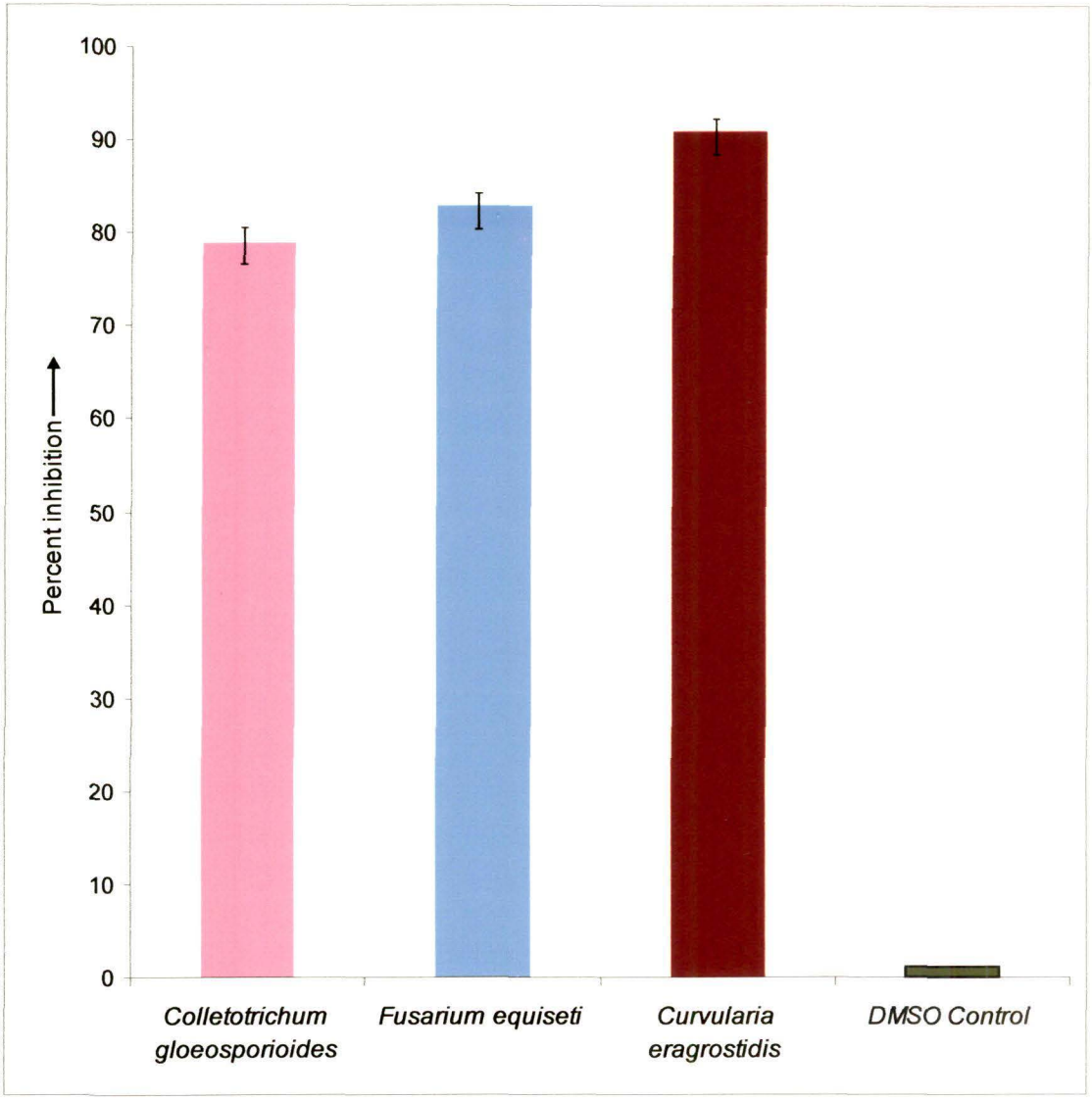


Fig. 18: Percent inhibition of spore germination at 300 ppm concentration of compound A (lupeol).

Table 2.9: Antibacterial activity of Lupeol (compound-A)

Bacterial organism	Concentrations of compound (ppm)	Diameter of Inhibition zone (cm)*
Ralstonia solanacearum	Control	-
	100	0.4
	200	0.6
	300	0.9
	400	1.2
	500	1.4
Xanthomonas sp	control	-
	100	1.5
	200	1.7
	300	1.9
	400	2.1
	500	2.2
Pseudomonas syringae	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.6
	500	1.7
Erwinia carotovora	Control	-
	100	1.1
	200	1.4
	300	1.7
	400	1.9
	500	2.2

*mean of three replications; - indicates no inhibition zone formed

Compound B, first derivative of mother compound lupeol identical as 2,2-dibromo lupanone obtained from the following reactions: by hydrogenation of lupeol, lupanol was prepared. From lupanol, lupanone was formed by Jone's oxidation. Treating lupanone with N-bromosuccinimide, in dimethylsulfoxide-chloroform mixture, compound-B(2,2–dibromo lupanone) was prepared.

Results of spore germination bioassay have been presented in table-2.10. From the results it was found that the antifungal activity of 2,2-dibromolupanone (compound-B) was effective at all the five concentrations. But three concentrations (300, 400 and 500ppm) showed significant antifungal activity (89-99% inhibition of spore germination) against the three fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *curvularia eragrostidis*). Percent germination of spores gradually decreased with the

increasing concentration of the compound. In all the three cases 500 ppm concentration was more effective than the other concentrations.

Table2.10: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 2,2 –dibromo lupanone (compound B) (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-48	23	77±1.10
	200	08-44	05	95±1.34
	300	08-40	03	97±1.58
	400	04-36	03	97±1.67
	500	04-28	01	99±1.87
Fusarium equiseti	100	12-20	27	73±1.60
	200	08-32	12	88±1.77
	300	08-32	11	89±1.98
	400	04-12	09	91±1.45
	500	04-12	06	94±1.65
Curvularia eragrostidis	100	36-100	13	87±1.20
	200	28-72	08	92±1.40
	300	24-68	07	93±1.44
	400	20-60	06	94±1.02
	500	16-44	04	96±1.19

*Data after ± indicate standard error value

From the results presented in the table 2.11 and plate-2 it was found that the antifungal activity of 2, 2 dibromolupanone (compound B) was very much significant. Out of five concentrations tested three concentrations (300,400,500ppm) of the compound B were very effective and showed antifungal activity (inhibition zones diameter of 1.0-2.0cm) against all the three tested fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti and Curvularia eragrostidis*). Diameter of inhibition

zones were increased with the increasing concentration of the compound. Best antifungal activity was obtained against all the three fungus when 500 ppm concentration was used.

In table 2.12 and plate-2 the results of antibacterial activity of 2,2-dibromolupanone (compound-B) against four bacteria (*R. solanacearum, Xanthomonas sp, P. syringae* and *E. carotovora*) have been presented. Out of five concentrations tested, three concentrations (300,400,500ppm) of the compound were proved to be significant (diameter of inhibition zones ranged from 1.2-2.3cm). The diameter of inhibition zones were gradually increased with increasing concentrations of the compound-B. Highest antibacterial activity was observed at 500ppm concentration of compound-B.

Table 2.11: Antifungal activity of 2,2 –dibromo lupanone (compound B)

Fungal organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Colletotrichum gloeosporioides	100	0.6
	200	0.8
	300	1.0
	400	1.2
	500	1.3
Fusarium equiseti	100	1.3
	200	1.5
	300	1.6
	400	1.7
	500	1.9
Curvularia eragrostidis	100	1.2
	200	1.5
	300	1.7
	400	1.8
	500	2.0

*mean of three replications

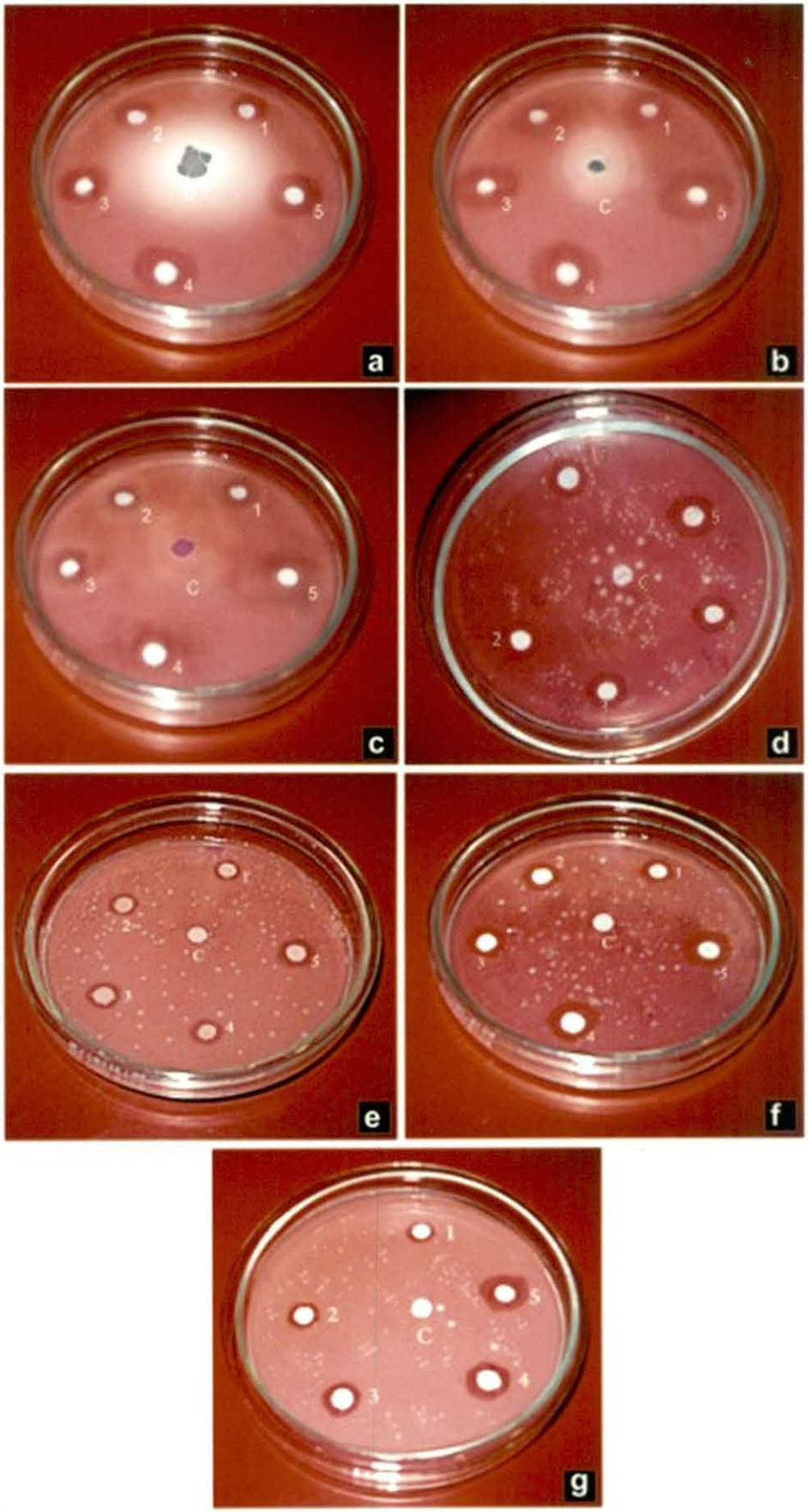


Plate 1: Disc diffusion test for anti microbial activity of compound A: (Lupeol) at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

* 1=100ppm, 2=200 ppm, 3=300 ppm, 4=400 ppm, 5=500ppm, c=DMSO control.

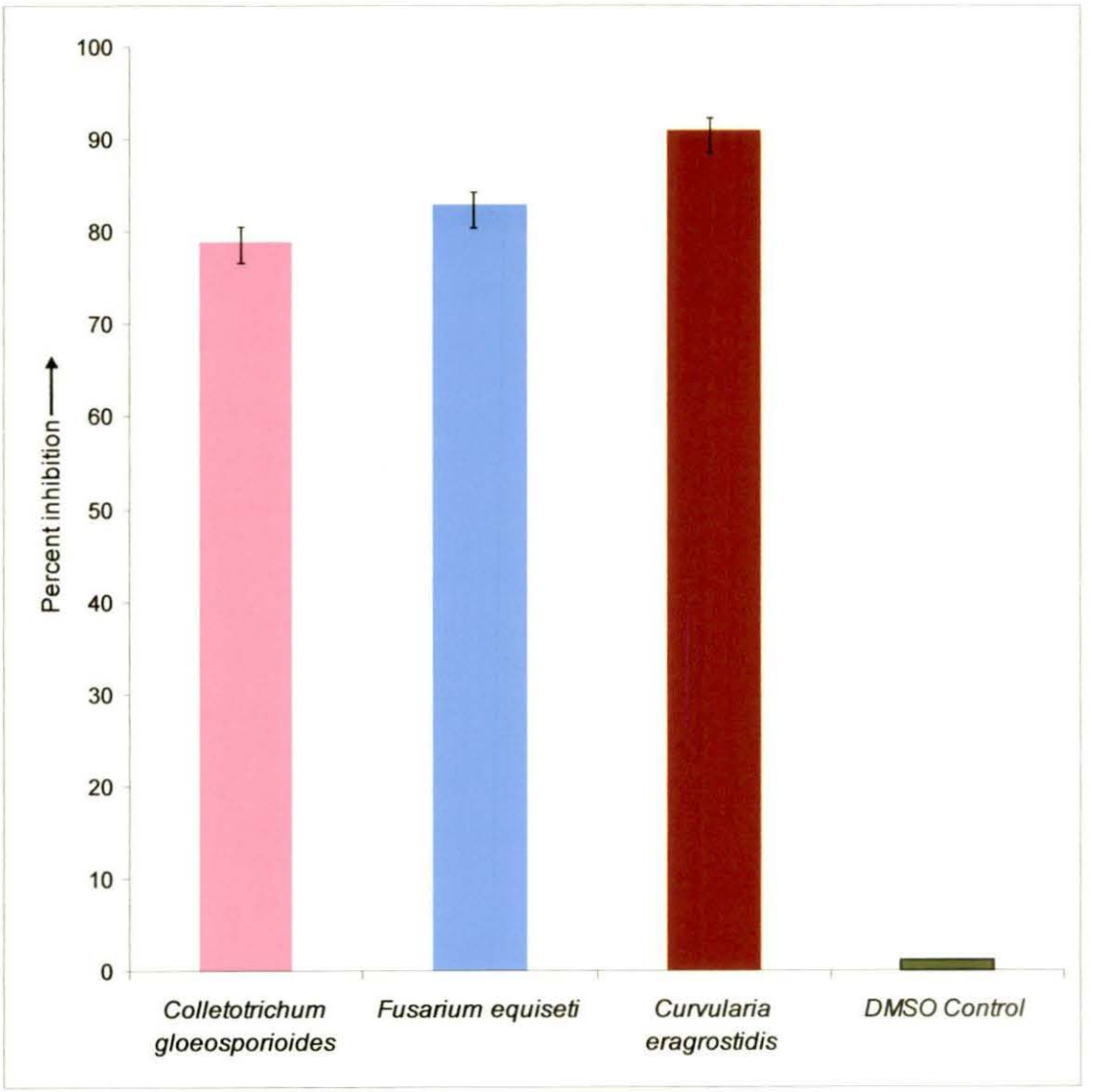


Fig. 18: Percent inhibition of spore germination at 300 ppm concentration of compound A (lupeol).

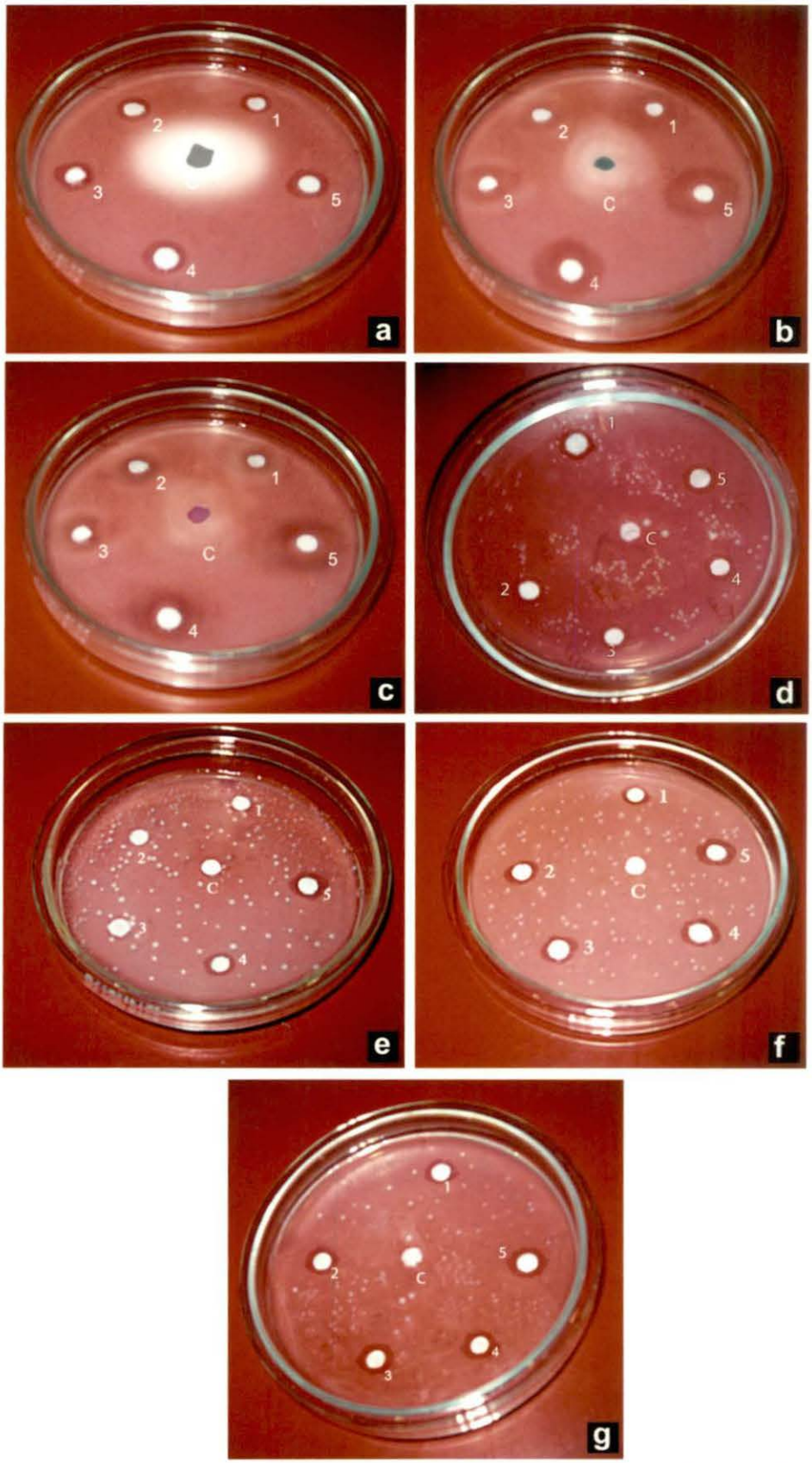


Plate 2: Disc diffusion test for anti microbial activity of compound B: (2,2-dibromo lupanone); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

* 1=100ppm, 2=200 ppm, 3=300 ppm, 4=400 ppm, 5=500ppm, c=DMSO control

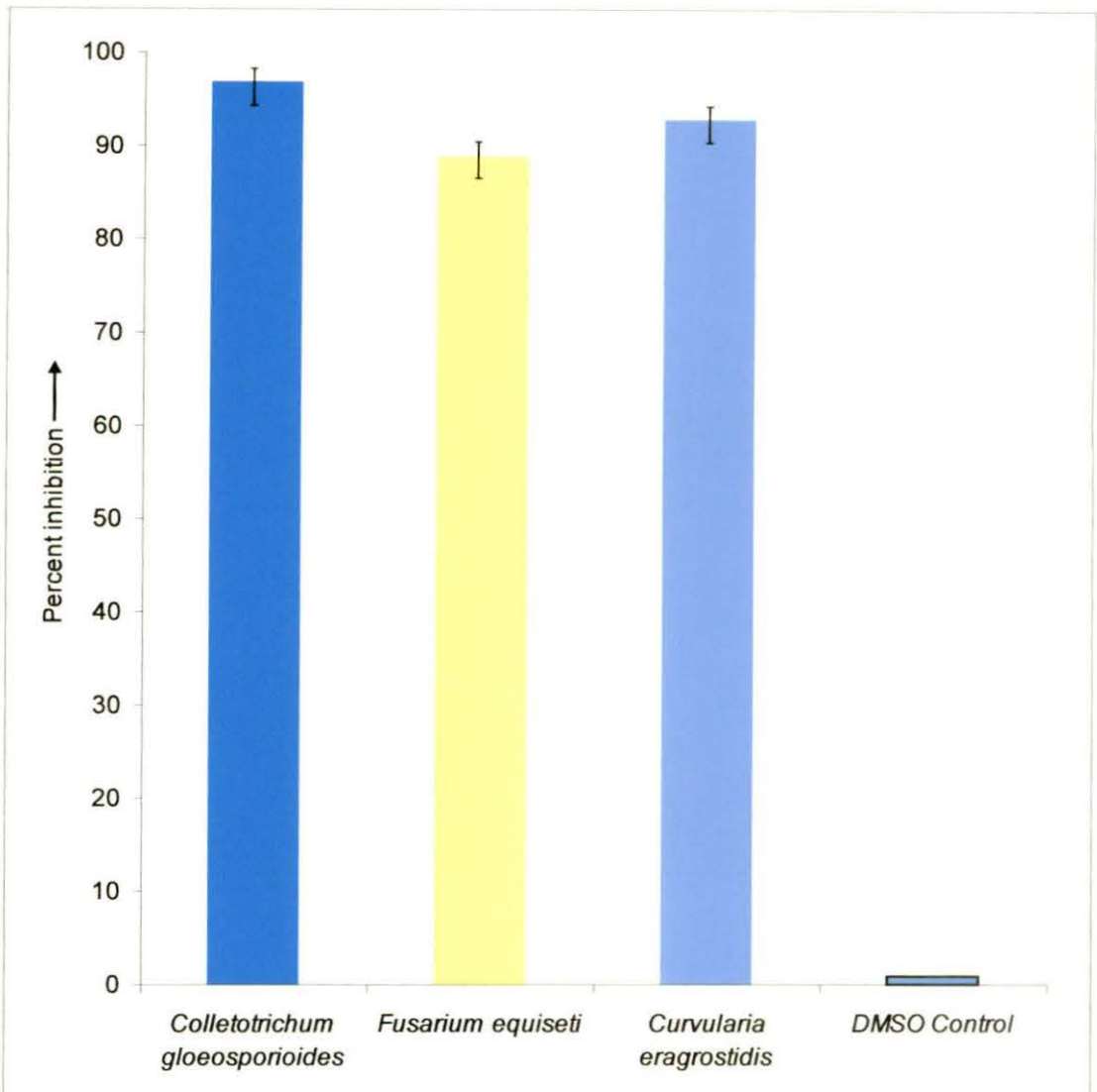


Fig. 19: Percent inhibition of spore germination at 300 ppm concentration of compound B (2,2-dibromolupanone).

Table 2.12: Antibacterial activity of 2,2–dibromo lupanone (compound B)

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone (cm)*
Ralstonia solanacearum	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.8
	500	2.0
Xanthomonas sp	control	-
	100	1.2
	200	1.4
	300	1.7
	400	1.9
	500	2.2
Pseudomonas syringae	Control	-
	100	0.8
	200	1.0
	300	1.2
	400	1.5
	500	1.7
Erwinia carotovora	Control	-
	100	1.2
	200	1.5
	300	1.8
	400	2.0
	500	2.3

*mean of three replications; - indicates no inhibition zone formed

Compound-C, the second derivative of lupeol was identified as 2α-bromolupanone. 2αbromolupanone was obtained from N-bromosuccinimide by treatment of lupanone in DMSO-$CHCl_3$ as mentioned in the chemical derivative preparation section. From the results, presented in the table 2.13 it was evident that the antifungal activity of 2α–bromolupanone (compound C) was effective in controlling plant

pathogens. In all the five concentrations tested compound-C showed antifungal activity against the three fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti and Curvularia eragrostidis*). Inhibition of spore germination was gradually increased with the increasing concentrations of the compound. Although all concentrations of the compound were effective but 500 ppm concentration showed more than 95% inhibition of spore germination.

Table 2.13: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 2α-bromolupanone (compound C) (when control raised to 100).

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-48	21	79±1.12
	200	12-48	17	83±1.79
	300	08-40	07	93±1.60
	400	04-36	06	94±1.77
	500	04-28	02	98±1.88
Fusarium equiseti	100	12-20	17	83±1.98
	200	08-16	12	88±1.75
	300	08-16	10	90±1.56
	400	04-12	06	94±1.36
	500	04-08	02	98±1.30
Curvularia eragrostidis	100	36-84	20	80±1.57
	200	32-72	12	88±1.10
	300	28-64	11	89±1.35
	400	24-56	10	90±1.90
	500	20-44	05	95±2.00

*Data after ± indicate standard error value

Antifungal activity of 2α-bromolupanone (compound-C) was also tested by disc diffusion method on agar plates. Results presented in the table 2.14 and plate-3 showed that compound-C was antifungal against *C. gloeosporioides* and *F. equiseti* but it was not effective against *C.eragrostidis.* Compound-C was tested for antifungal activity at five

different concentrations (100,200,300,400,500ppm). Compound-C at concentration below 300ppm did not show any antifungal activity. Diameter of inhibition zones ranged from 0.8-2.1 cm when concentrations of 300,400 and 500ppm of the compound-C were tested against the two pathogens mentioned above.

The results of antibacterial activity of 2α–bromolupanone (compound-C) presented in table 2.15 and plate-3 indicated that Compound-C was effective against three bacteria (*R. solanacearum, Xanthomonas sp, E.carotovora*). Out of five tested concentrations, two concentrations (400 and 500ppm) of the compound were proved to be significant against all three bacteria.It was evident from the results that compound-C was very less effective against *P. syringae.* Only 500ppm concentration of the compound-C showed its activity against *P. syringae.*

Table 2.14: Antifungal activity of 2α-bromolupanone (Compound-C)

Fungal organism	Concentrations of Compound (ppm)	Diameter of inhibition zone (cm)*
Colletotrichum gloeosporioides	100	-
	200	-
	300	0.8
	400	1.2
	500	1.4
Fusariumequiseti	100	1.5
	200	1.5
	300	1.6
	400	2.0
	500	2.1
Curvularia eragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

*mean of three replications; - indicates no inhibition zone formed

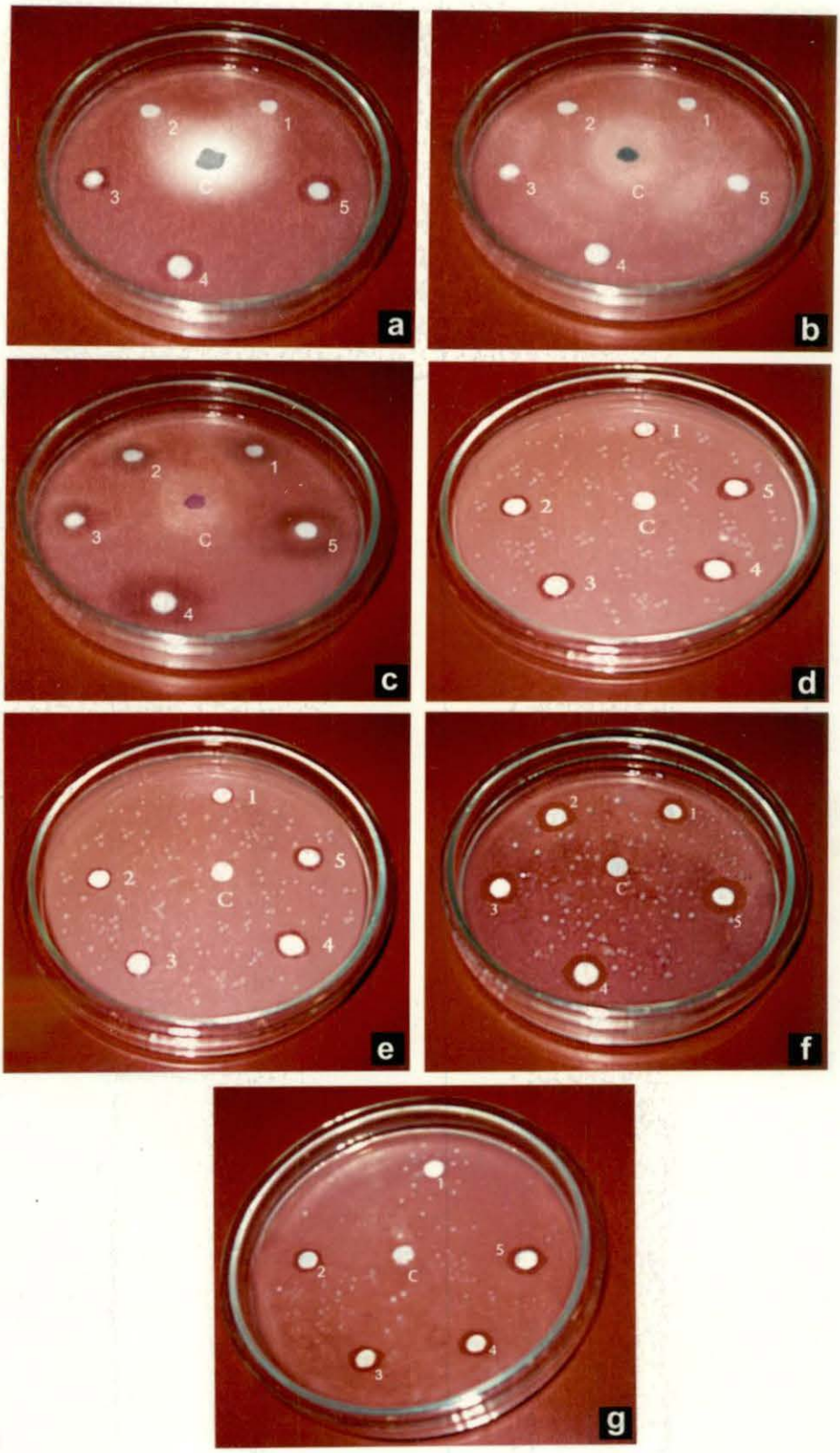


Plate 3: Disc diffusion test for anti microbial activity of compound C: (2α bromo lupanone); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

* 1=100ppm, 2=200 ppm, 3=300 ppm, 4=400 ppm, 5=500ppm, c=DMSO control.

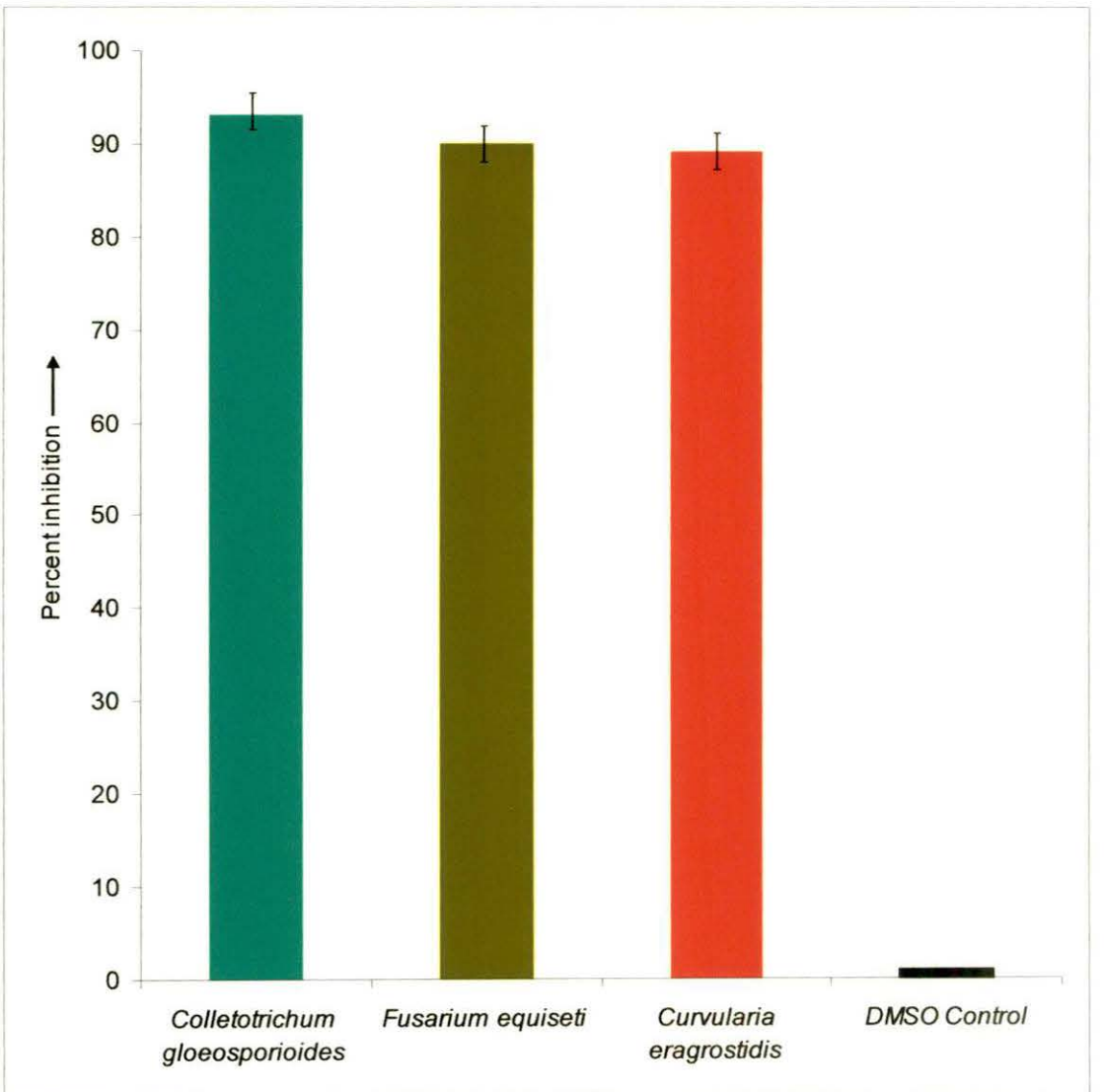


Fig. 20: Percent inhibition of spore germination at 300 ppm concentration of compound C (2α-bromolupanone).

Table 2.15: Antibacterial activity of 2α-bromolupanone (Compound "C")

Bacterial organism	Concentrations of compound (ppm)	Diameter ofinhibition zone (cm) *
Ralstonia solanacearum	Control	-
	100	0.4
	200	0.4
	300	0.8
	400	1.0
	500	1.3
Xanthomonas sp	control	-
	100	0.4
	200	0.8
	300	1.0
	400	1.2
	500	1.3
Pseudomonas syringae	Control	-
	100	0.4
	200	0.4
	300	0.4
	400	0.9
	500	1.1
Erwinia carotovora	Control	-
	100	0.7
	200	0.9
	300	1.2
	400	1.3
	500	1.5

*mean of three replications; - indicates no inhibition zone formed

Compound-D, the third derivative of mother compound lupeol, (identical as lup-1(2)-en-3-one) obtained from the reaction: Dehydro bromination of 2α-bromo lupanone with lithium bromide-N,N-dimethyl formamide.

From the results presented in the table 2.16 it was found that the antifungal activity of lup-1(2)-en-3-one (Compound-D) was positive. Out of five concentrations

tested three concentrations (300,400,500ppm) of the compound D were very much effective and showed antifungal activity (89-97% inhibition of spore germination) against all the three fungal pathogens (*Colletotrichum gloeospoerioides,Fusarium equiseti and Curvularia eragrostidis*) tested. Percent germination was reduced gradually with increasing concentration of the compound. Although 300,400 and 500ppm concentrations of the compound were effective but 500 ppm concentrations was most effective.

Table 2.16: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by Lup-1(2)-en-3-one (Compound"D"). (when control raised to 100)

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	26	74±1.97
	200	08-40	12	88±2.01
	300	08-36	11	89±1.85
	400	04-32	08	92±1.66
	500	04-28	04	96±1.44
Fusarium equiseti	100	08-24	25	75±2.00
	200	08-20	15	85±1.73
	300	08-16	10	90±1.32
	400	04-16	03	97±1.56
	500	04-12	02	98±1.40
Curvularia eragrostidis	100	40-96	13	87±1.75
	200	32-80	11	89±1.30
	300	28-52	09	91±1.99
	400	24-48	06	94±1.45
	500	20-32	05	95±1.77

*Data after ± indicate standard error value

From the results presented in the table 2.17 it was found that the antifungal activity of **Lup-1(2)-en-3-one** (Compound-D) was very much effective against the pathogens tested. Diameter of inhibition zones ranged between 0.9-2.5cm against all the three tested fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*). Diameter of inhibition zones was increased with the increasing concentration of the compound.

Results of antibacterial activity of Lup-1(2)-en-3-one (Compound-D) through disc diffusion method have been presented in table 2.18. Compound- D showed significant activity in connection of 300ppm concentration and above. Diameter of inhibition zones of the effective concentrations ranged from 0.9-1.5cm against the four test pathogens (*R. solanacearum, Xanthomonas sp, Pseudomonas syringae, E. carotovora*).

Table 2.17: Antifungal activity by Lup-1(2)-en-3-one (Compound"D")

Fungal organism	Concentrations of Compound (ppm)	Diameter of inhibition zone (cm)*
Colletotrichum gloeosporioides	100	1.4
	200	1.6
	300	1.7
	400	1.9
	500	2.1
Fusarium equiseti	100	1.9
	200	2.0
	300	2.2
	400	2.3
	500	2.5
Curvularia eragrostidis	100	0.9
	200	0.9
	300	1.1
	400	1.2
	500	1.4

*mean of three replications

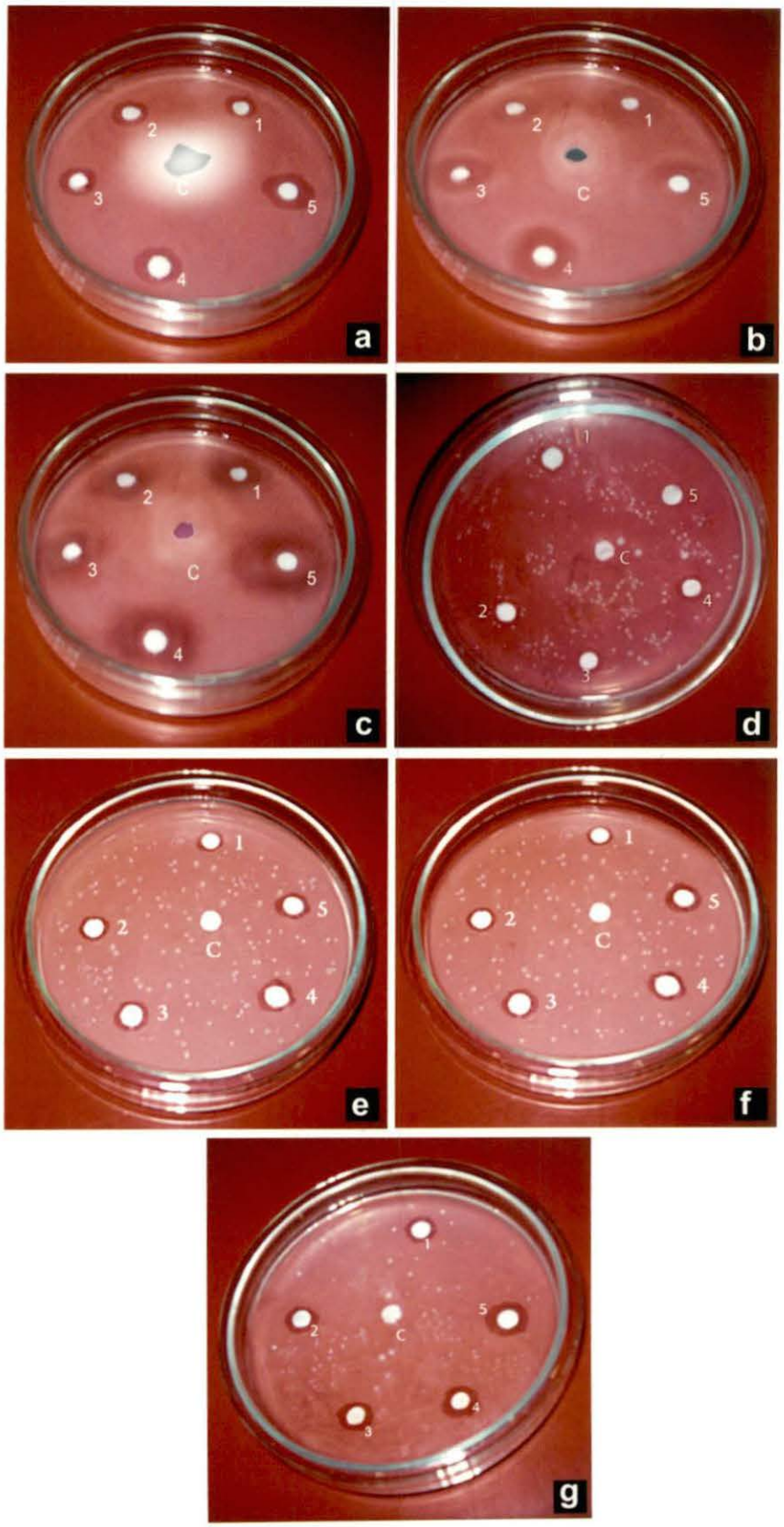


Plate 4: Disc diffusion test for anti microbial activity of compound D: (lup-1-2-en-3-one); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

* 1=100ppm, 2=200 ppm, 3=300 ppm, 4=400 ppm, 5=500ppm, c=DMSO control.

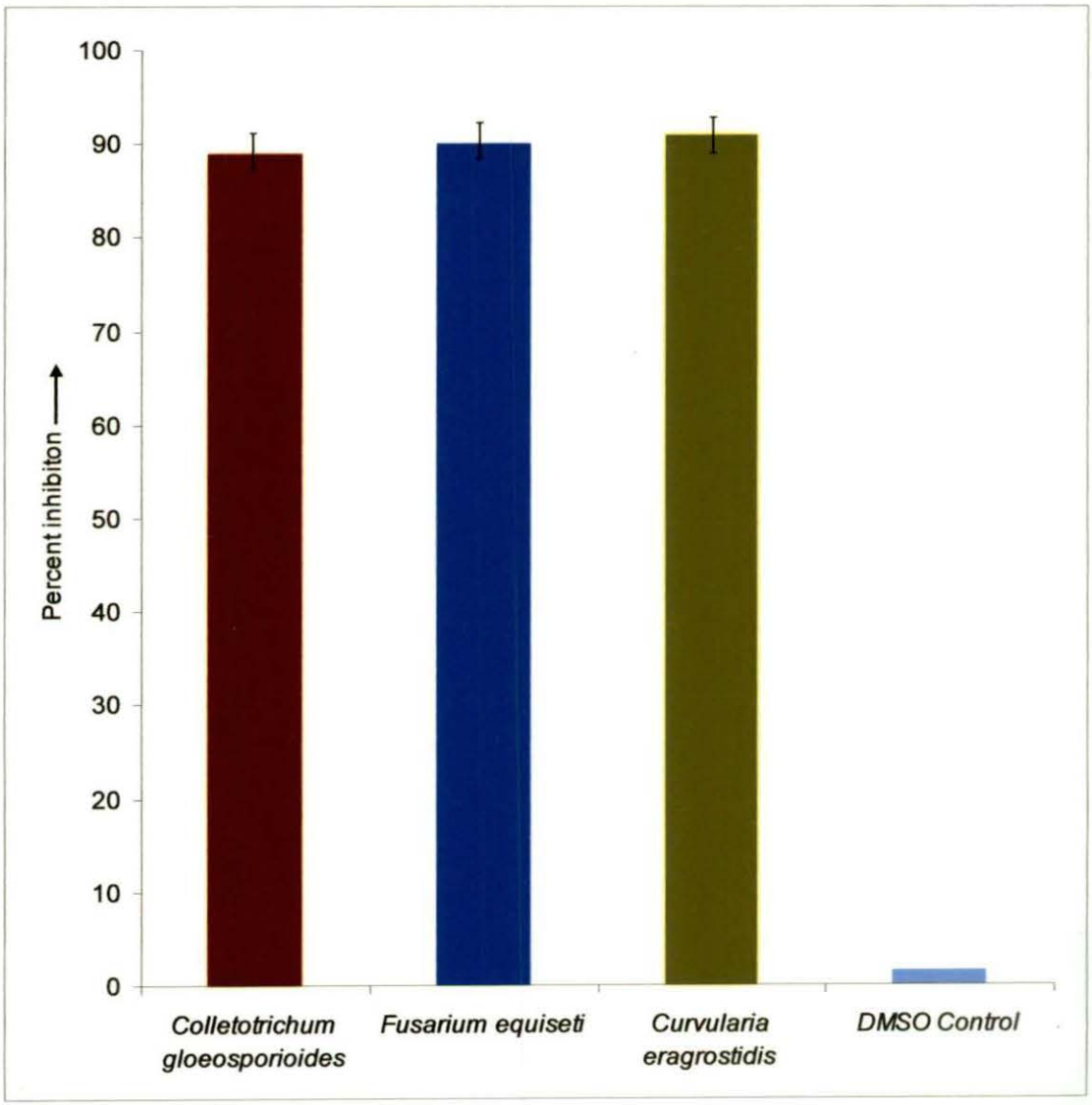


Fig. 21: Percent inhibition of spore germination at 300 ppm concentration of compound D (Lup-1-(2)-en-3-one).

Table 2.18: Antibacterial activity of Lup-1(2)-en-3-one (Compound"D")

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone (cm)*
Ralstonia solanacearum	Control	-
	100	0.4
	200	0.6
	300	0.9
	400	1.1
	500	1.3
Xanthomonas sp	control	-
	100	0.6
	200	0.8
	300	1.1
	400	1.2
	500	1.5
Pseudomonas syringae	Control	-
	100	0.4
	200	0.7
	300	0.9
	400	0.9
	500	1.2
Erwinia carotovora	Control	-
	100	0.7
	200	0.8
	300	1.0
	400	1.2
	500	1.4

*mean of three replications; - indicates no inhibition zone formed

Lupan-[2,3-C]-1',2',5'-oxadiazole (compound-E) was prepared following the chemical methods as described earlier. Compound-E was subjected to bioassays. The results of the spore germination bioassay of compound-E have been presented in Table

2.19. All the five concentrations tested were effective in controlling spore germination of the three fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*). Lowest concentration (100ppm) of the compound-E reduced 87%, 88% and 90% of spore germination in case of *C. gloeosporioides, F. equiseti* and *C. eragrostidis* respectively.

Table 2.19: Percent inhibition of spore germination of *Colletotrichum gloeosporioids, Fusarium equiseti* and *Curvularia eragrostidis* by lupane-[2,3-C]-1′,2′,5′-oxadiazole (Compound-E) (when control raised to 100)

Fungal organism	Concentrations of compound (ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	08-64	10	90±1.54
	200	08-56	05	95 ±1.90
	300	08-40	04	96 ±1.22
	400	08-36	01	99 ±1.47
	500	08-24	-	100 ±1.00
Fusarium equiseti	100	08-20	13	87 ±1.82
	200	08-20	09	91 ±1.59
	300	04-16	06	94 ±1.32
	400	04-12	03	97 ±1.77
	500	04-12	01	99 ±1.50
Curvularia eragrostidis	100	40-120	12	88 ±1.60
	200	36-88	07	93 ±1.66
	300	28-64	03	97 ±1.80
	400	16-40	02	98 ±1.99
	500	16-32	00	100 ±1.00

*Data after ± indicate standard error value; - indicates no fungal spore formed

Antifungal and antibacterial activity of lupane-[2,3-C]-1′,2′,5′-oxadiazole (compound E) were also tested against growth of three different fungal pathogens (*Colletotrichum gloeosporioides, Fusarium equiseti and Curvularia eragrostidis*) and four bacterial pathogens (*R.solanacearum,Xanthomonas sp.,P.syringae* and

E.carotovora). The results of the experiments have been presented in table 2.20, table-2.21 and plate-5. From the results, it was found that the antifungal activity of Compound-E was also very effective. All the five concentrations of the compound-E could significantly check the growth of *C. gleosporioides* and *F. equiseti.* But growth of *C. eragrostidis* was cheked by the compound only at 300ppm concentration and above.

In case of antibacterial test (table 2.21) of the compound it was found that growth of *Pseudomonas* and *Xanthomonas* was better controlled than the other two bacteria tested (*R.solanacearum* and *E.carotovora*). However, at highest concentration (500ppm) of the compound growth of all the four bacteria were controlled.

Table 2.20: Antifungal activity of lupane[2,3-C]-1′,2′,5′--oxadiazole (compound E)

Fungal organism	Concentrations of compound (ppm)	Diameter of inhibition zone* (cm)
Colletotrichum gleosporioides	100	1.6
	200	1.8
	300	2.0
	400	2.1
	500	2.3
Fusarium equiseti	100	1.4
	200	1.5
	300	1.6
	400	1.9
	500	2.0
Curvularia eragrostidis	100	-
	200	-
	300	0.6
	400	0.8
	500	1.0

*mean of three replications; - indicates no inhibition zone formed

Table 2.21: Antibacterial activity of lupane[2,3-C]-1′,2′,5′-oxadiazole(compound E)

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone* (cm)
Ralstonia solanacearum	Control	-
	100	0.6
	200	0.9
	300	1.0
	400	1.2
	500	1.5
Xanthomonas sp.	control	-
	100	1.3
	200	1.5
	300	1.8
	400	2.0
	500	2.2
Pseudomonas syringae	Control	-
	100	1.0
	200	1.3
	300	1.6
	400	1.8
	500	2.0
Erwinia carotovora	Control	-
	100	0.6
	200	0.8
	300	1.2
	400	1.5
	500	2.0

*mean of three replications; - indicates no inhibition zone formed

Phytotoxicity test of Lupeol and its derivatives:

Phytotoxicity of the five compounds was tested in tomato plants (of Priya variety popularly cultivated in the present study area). To test phytotoxicity, tomato plants were grown in earthen pots [size 15cm (diameter) and 15 cm (height)]. The pots were

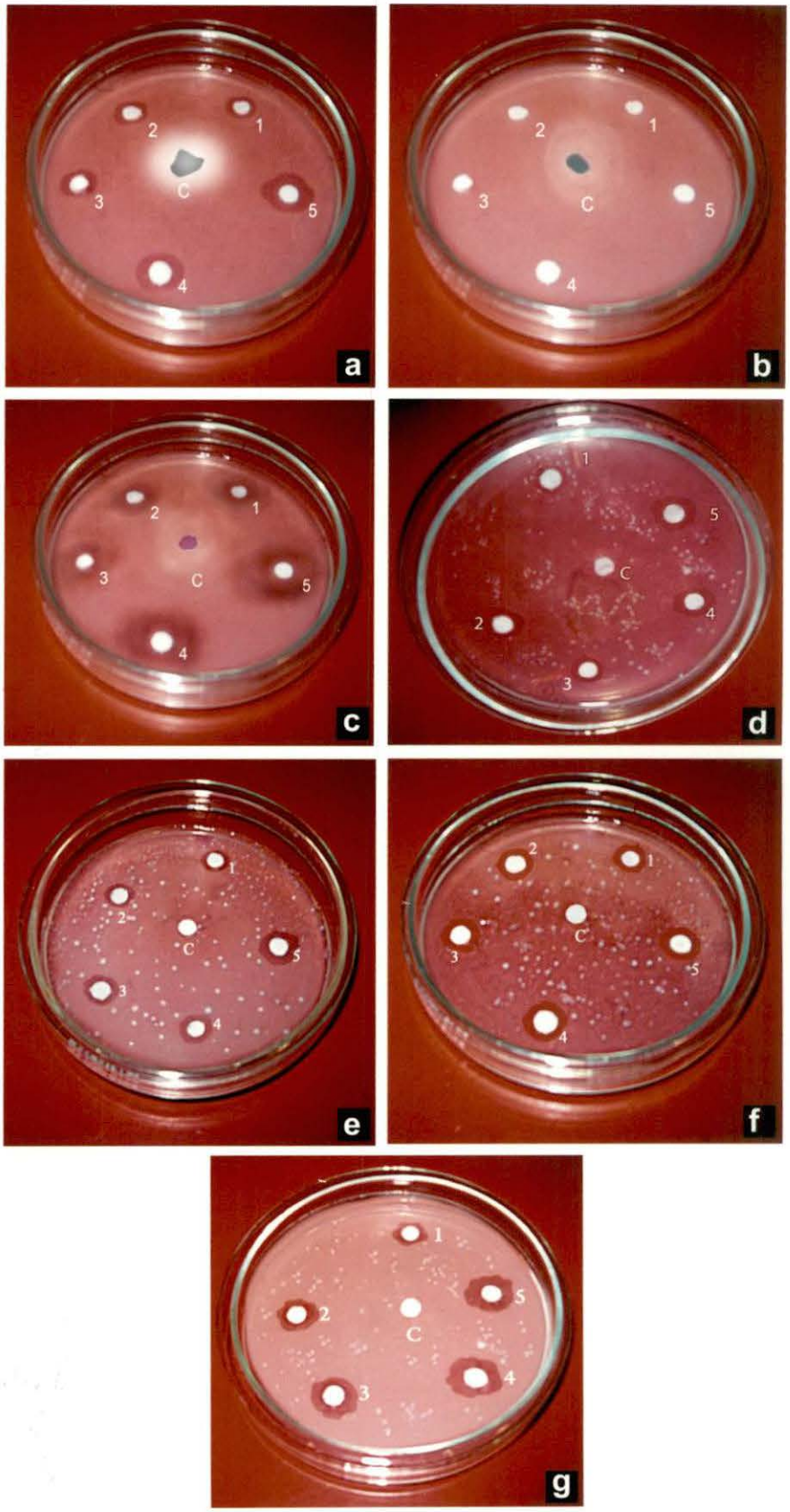


Plate 5: Disc diffusion test for anti microbial activity of compound E: (Lupane-[2,3-C]-**1′,2′,5′**-oxadiazole); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

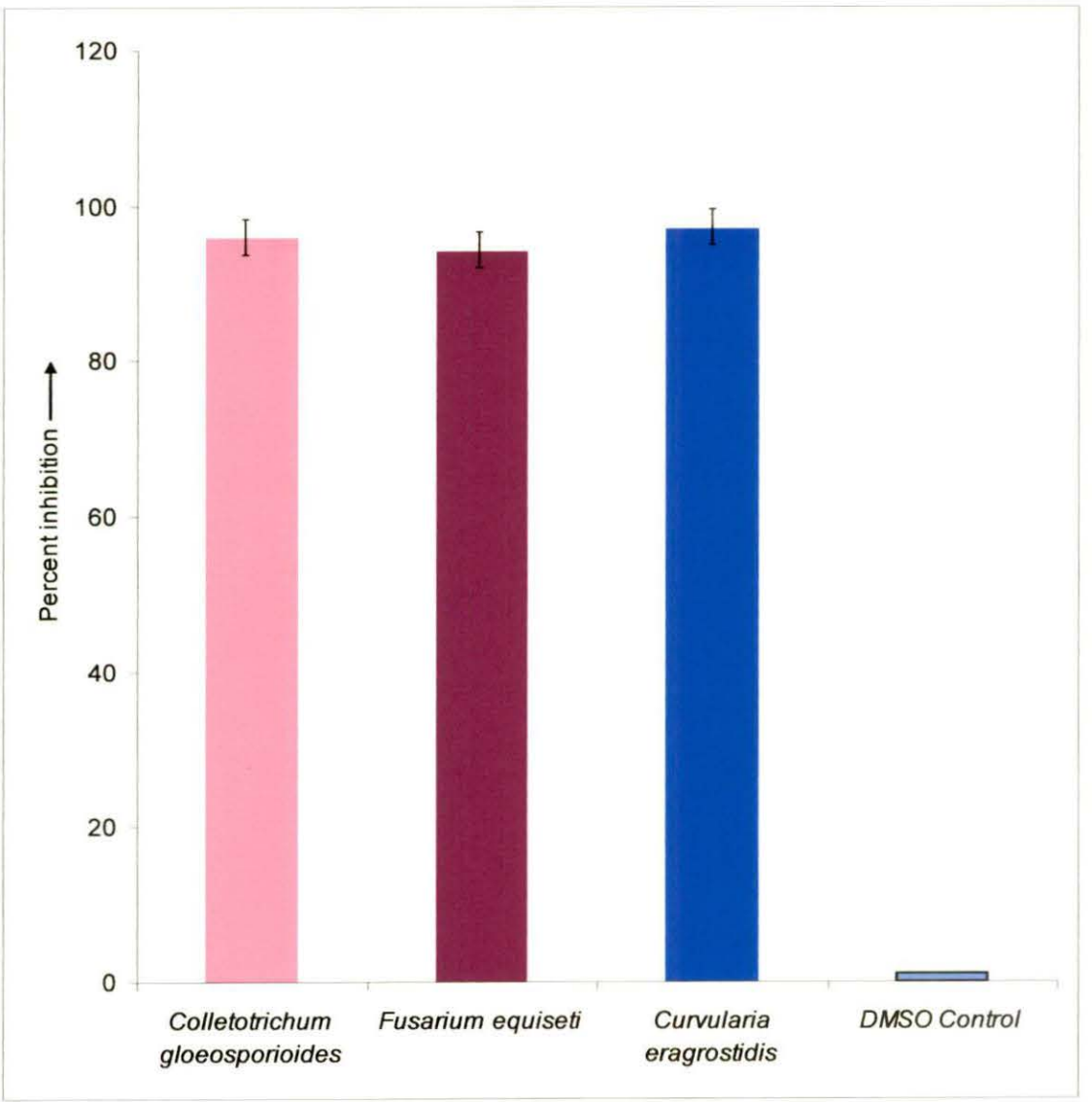


Fig. 22: Percent inhibition of spore germination at 300 ppm concentration of compound E (Lup [2,3-c]-1′,2′,5′-oxadiazole).

maintained in the experimental garden of the Department of Botany with normal watering. Sixty such pots containing plants (each 10 cm in height) were kept in a net house. The plants were grouped into six sets and each set contained 10 plants each. Five compounds (at 100 ppm concentration) were sprayed in five sets separately and the sixth set was sprayed with sterile distilled water. The plants were observed upto seven days. No phytotoxicity was observed at 100ppm concentration of the compounds when compared with the plants of control set which were sprayed with the sterile distilled water. (table-2.22)

Table 2.22: Phytotoxicity of lupeol and its four derivatives

Compounds	Concentrations (ppm)	Morphological & Physiological abnormalities abnormalities			
		1day	3day	5day	7day
A	100	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated
B	100	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated
C	100	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated
D	100	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated
E	100	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated
Control	Sterile distilled water	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated	Plants alive, no significant changes Root-germinated Shoot- germinated

2.3: DISCUSSION

The present study was based on the phytochemical investigations and antimicrobial activities of the plant-*Xanthoxylum budrunga.* This plant contains medicinal as well as anti-microbial properties. Lupeol, a triterpenoid (special group of secondary metabolites) was the mother compound isolated from the plant.

Triterpenoid lupeol containing a C30 skeleton was isolated and characterized from *X. budrunga* plant of proven anti-inflammatory, anti-tumor and antimicrobial activity.[5,13] Saleem *et al.*(2001) reported that Lupeol, a triterpene, inhibits early responses of tumor promotion induced by benzoyl peroxide in murine skin. Malini *et al.*(2000) also reported that protective effect of triterpenes on calcium oxalate crystal induced peroxidative changes in experimental urolithiasis.

The stem bark of the plant was used to treat cough, cold, toothache, infected wounds, chest pain etc. [3] Fernandez *et al.* (2001) revealed a new insights into the mechanism of action of the anti-inflammatory triterpene lupeol. Yadava and Chakravarti (2008) reported anti-inflammatory activity of a new triterpenoid saponin from *Carthamus tinctorius* whose mechanism of action was similar to lupeol. Commercially the plant is used to make heavy flooring, furniture, agricultural instruments *etc*[4]. The medicinal properties deserve more attention because of the claimed antimicrobial activities which have been confirmed by pharmacological research [7].

In the present study four derivatives of isolated triterpenoid (lupeol) were prepared and characterized. Bioassays of all the five natural or derived compounds were performed against some fungal and bacterial pathogens of economically important plants. Lupeol (compound A), the mother compound was extracted from dried and powdered trunk bark of the plant *Xanthoxylum budrunga* with chloroform in Soxhlet apparatus for 20 hours. In these extraction process different compounds were obtained through column chromatographic separation. From the fractions obtained lupeol was identified and characterized. From lupeol, an intermediate derivative called lupanol was prepared by hydrogenation process and were characterized. Another intermediate derivative lupanone was prepared from lupanol by Jone's oxidation process. In Jone's oxidation different fractions were obtained from column chromatographic separation and the derivatives

were characterized. Lupanone treated with N-Bromosuccinimide form 2,2-dibromolupanone (compound B) and 2α-bromolupanone (compound C). Both compound-B and compound-C were characterized. Another derivative of lupeol named lup-1(2)-en-3-one (compound D) was prepared by dehydrobromination of 2α-bromolupanone with lithium bromide–N,N-dimethyl formamide and was also characterized. Lupan-[2,3-C]-1',2',5'-oxadiozole, called compound-E, the fourth derivative of lupeol was prepared by cyclisation of 2,3-dioximelupane in dry DMF under microwave irradiation (100w,100^{0}C) for 10 mins.

To observe antimicrobial activity of lupeol and its derivatives three fungi *viz. Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* and four bacteria *viz. Ralstonia solanacearum, Xanthomonas sp., Pseudomonas syringae* and *Erwinia carotovora* were used in the present study. All the five chemicals were tested for their phytotoxicity, if any, on tomato plants.

The biological activity of natural lupane derivatives was reliably established [34]. In the last decade, interests in the pharmacological properties of lupane derivatives were observed after revealing promising antiviral (particular Anti-HIV) and antineoplastic agents among these compounds [35]. Antifungal and antimicrobial activities of lupeol was also reported [16].

The compound–A, chemical name 'Lupeol' showed antibacterial activity and anti fungal activity.. In disc diffusion studies growth of two fungi (*Colletotrichum gloeosporioids, Fusarium equiseti)* were controlled by compound-A. *Curvularia eragrostidis* could not be controlled in disc diffusion test even at 500ppm concentration of lupeol. Lupeol showed little activity against *Ralstonia solanacearum* at lowest concentration (100ppm). In contrast, three other bacteria (*Xanthomonas sp, P. syringae, E. carotovora*) tested were controlled significantly at 100ppm concentration of lupeol.

Compound–B (2,2dibromolupanone) a derivative of compound-A was stronger than the mother compound Lupeol, as evident from the result of spore germination bioassay and disc diffusion bioassay against three different fungi (*Colletotrichum gloeosporioids, Fusarium equiseti* and *Curvularia eragrostidis).* In contrast to the mother compound (lupeol) which could not check the growth of *Curvularia eragrostidis* in disc

diffusion test, compound-B could control *Curvularia eragrostidis* at all the five concentrations (100, 200, 300, 400 & 500ppm) tested. Stronger antibacterial activity of compound-B was also evident fron the disc diffusion test against the four bacterial pathogens (*R.solanacearum, Xanthomonas sp., P.syringae* and *E.carotovora*).

The second derivative of lupeol coded as compound-C (2α-bromolupanone) was tested for antifungal and antibacterial activity. Compound-C could check spore germination of all the three fungi (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*) moderately at five different concentrations of the compound tested. In disc diffusion test the compound showed no activity against *Curvularia eragrostidis*. The compound showed poor activity against *Colletotrichum gloeosporioides* but showed high activity against *Fusarium equiseti*. In case of bacteria only *E. carotovora* was checked at 100ppm concentration. Growth of all three other bacteria (*Ralstonia solanacearum, Xanthomonas sp.& Pseudomonas syringae*) were controlled only at higher concentrations of the compound-C. Thus compound-C was less antimicrobial than compound-B.

The third derivative of lupeol presently coded as compound-D (lup-1(2)-en-3-one) showed moderate antifungal activity both in case of spore germination bioassay and disc diffusion bioassay. In higher concentrations of the compound all the four bacteria (*R. solanacerum, Xanthomonas sp., P. syringae* and *E. carotovora*) were controlled significantly as evident from the result presented in table-2.16. In lower concentrations of compound-D, *Xanthomonas* sp and *E. carotovora* were controlled but it could not control growth of *R. solanacerum* and *P. syringae*.

The fourth derivative of lupeol coded as compound-E (Lupane[2,3-C]-1',2',5'-oxadiozole) was also subjected to spore germination bioassay and the results (presented in table-2.19) showed good antifungal activity when tested in spore germination bioassay. Inhibition of spore germination ranged between 80-100% in the spore germination bioassay. In disc diffusion bioassay 100ppm and 200ppm concentration of compound-E showed no antibacterial inhibition zone on the plates. However, higher concentrations of compound-E could control the three bacteria significantly even at lowest concentration tested (100ppm). All the four bacteria showed moderate to high antibacterial activity

respectively with the increasing concentrations (100ppm-500ppm) of the compound-E. Thus the compound showed good antibacterial activity and moderate antifungal activity.

Similar studies were also done by Shai *et al.* (2008) who isolated four compounds lupeol (1), betulinic acid (2), ursolic acid (3) and 2-alpha-hydroxyursolic acid (4) from the leaves of *Curtisia dentata*. They studied the antibacterial and antifungal activity using broth microdilution assay and bioautography method. Mansouri (1999) found new antibacterial agents from ethanolic extracts of ten plants. The extracted compounds from the plants were screened for antibacterial activity. The agents were effective against *Staphylococcus aureus*. Among the plants *Mentha vividis L.* was also active against the isolates and inhibited the growth of 48.7% of the isolates. Kamalakanman *et al.* (2009) extracted 20 plant leaves and screened their inhibitory effect against the rice blast pathogen. They reported that *Prosopis juliflora* followed by *Zizyphus jujube* and *Abutilon indicum* significantly inhibited the mycelial growth and biomass as well as toxin production and spore germination under laboratory conditions. Ragasa *et al.* (2001) extracted the air dried leaves of *Vitex negundo* which afforded vitexilactone and casticin by silica gel chromatography. Their structures were elucidated by extensive 1D and 2D NMR spectroscopy. They studied their activity and found antifungal activity (against *Candida albicans* and *Aspergillus niger*) and antibacterial activity (against *Staphyllococcus aureus* and *Pseudomonas aeruginasa*). They also reported inactivity of the compounds against *Escherichia coli* and *Bacillus subtilis*. Hence, it was necessary to screen antimicrobial activity of the basic triterpenoid compound and its derivatives. From our studies, thus, antimicrobial activity of lupeol and its derivatives against some fungus and bacteria were significantly screened. Saleh *et al.* (1999) also isolated the known triterpenoids lantic acid, camarinic acid and lantanilic acid from *Lantana camara* (L) cultivated in Egypt and carried out the antibacterial activity of lantic acid using bioautography assays for Gram-positive and Gram-negative bacteria. They also compared their doses to 0.05 and 0.005 μg for chloramphenicol. They showed that lantic acid has broad spectrum antibacterial activity and may hold potential as a non-selective antimicrobial agent. Khan *et al.* (2008) isolated amblyone, a triterpenoid from *Amorphophallus campanulatus* and studied *in vitro* antibacterial, antifungal and cytotoxic

activities using disc diffusion technique. The minimum inhibitory concentration of amblyone was determined using serial dilution technique. They observed large zones of inhibition in disc diffusion test. They performed antibacterial screening against four Gram-positive bacteria (*Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus* and *Streptococcus pyogenes*) and six Gram-negative bacteria (*Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella flexneri, Pseudomonas aeruginosa* and *Salmonella typhi*). The MIC values determined against those bacteria were ranged between 8 to 64 µg/ml. In antifungal screening, the compound showed small zones of inhibition against *Aspergillus flavus, Aspergillus niger, Rhizopus aryzae*, and *Candida albicans*. Thus fungi were resistant against the compound. Similarly, our results also showed the susceptibility and resistance of the pathogens against the six compounds tested. The effective and nonphytotoxic concentration (100 ppm) was comparatively low than the concentrations tested by other authors. Thus the significance of the study was established.

On the basis of phytotoxicity tests, it was found that 100ppm concentration of compounds A, B, C, D, and E were not phytotoxic. From the present study, it was evident that several plant pathogens could be controlled by the compounds at 100 ppm concentration (non phytotoxic). Hence, the effective compounds may be recommended for controlling the respective plant pathogens *in vivo* or in field conditions.

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