■ CHAPTER - III

Title: Extraction and characterization of Betulinic acid from *Bischofia javanica*, preparation of its derivatives and antimicrobial activity of each of them

Several medicines have been extracted from plants since pre-historic ages. Certain alkaloids, steroids, terpenoids, and the secondary metabolites functioned as defensive agents against harmful pathogens [1]. It was also assumed that these compounds were used to treat human aliments. Following this, people from all over the world made some attempts to isolate these classes of natural products from the medicinal plants and evaluated their biological activities [2]. In the present chapter, the antimicrobial properties of the plant *Bischofia Javanica* have been discussed.

Bischofia javanica is an evergreen or semi evergreen woody tree with a maximum height of 40 meter and diameter of 2.3 meter. The relative short trunk is erect, branches low. The bark in nearly smooth, grayish brown, 1 cm thick and contains a red milky sap that become resinous and semi solid when dried. The male inflorescence is 8-13 cm long and pubescent to glabrous, while the female inflorescence is 15-17 cm long and pendent. Appearing in August to October, berry like fruits are light brown, globular or sub globular 6-13 cm in diameter, containing oblong seeds 5mm in length [5]. The plant *Bischofia javanica* belongs to the family Euphorbiaceae. *Bischofia* is a very small member of the family Euphorbiaceae and contain only two species distributed from southern Asia to Australia. They also occur in south-western, central and eastern part of China. In India it is found only in north Himalayan region [3].

The wood of *Bischofia javanica* is red, heavy, hard, and fine grained and used as a material for buildings. The fruits are used in making wine. Containing 30-45% oil, the edible seed is a source of red dye. The roots are used for medicinal properties. It also showed antifungal activities [4].

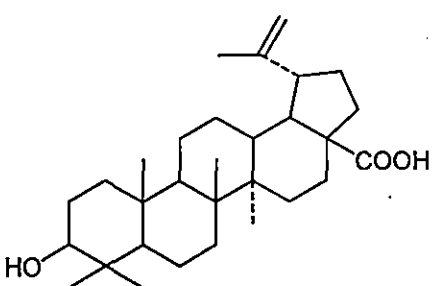
The ethanol extract of the bark of *Bischofia javanica* was partitioned in petroleum ether and dichloromethane then with ethyl acetate. The crude ethnolic extract and several

partitioned fractions showed broad spectrum anti-bacterial activities [6]. Betulinic acid and its derivatives were isolated from Bioassay guided fractionations of the $CHCl_3$ extract of the outer bark of *Bischofia javanica*.

Section 1: Extraction and characterization of pentacyclic triterpenoid (Betulinic acid) from *Bischofia javanica*.

1.1. Extraction from outer bark of *Bischofia javanica*: Isolation of Betulinic acid.

Dried and powdered trunk bark (3 kg) of *Bischofia javanica* was extracted with ethylacetate in Soxhlet apparatus for 72 hours. Ethyl acetate was distilled off and the gummy residue (14 g) was taken up in ether (1.0 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 aqueous layer was acidified (1 lt) when some insoluble solids separated out. The acidified portion was extracted with ether, washed with water until neutral and then dried using anhydrous Na_2SO_4. Ether was removed when a gummy residue (10 g) of crude betulinic acid was obtained and purified by column chromatography. Suitable mixture of petroleum ether and ethylacetate were used as eluent and crystallized from chloroform-methanol afforded betulinic acid of m.p 299^0—302^0C.



(71)
Betulinic acid

1.2. Estarification of Betulinic acid: Isolation of Methylbetulinate

To the crude betulinic acid (10 g) dissolved in ether (600 mL), a solution of diazomethane in ether [prepared from *N*-nitrosomethyl urea (5 g)] was added at cold and

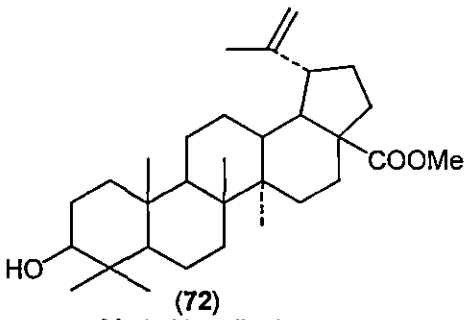
the reaction mixture was kept overnight. Next day excess of diazomethane was destroyed with glacial acetic acid (2 mL) .The ether solution was washed with water, then 10% sodium bicarbonate solution and again with water until neutral and dried by using anhydrous Na_2SO_4. Evaporation of the ether yielded a gummy residue (4.5 g). This crude ester dissolved in petroleum ether (15mL) was placed over a column of silica gel (60-120 mesh, 30 g) developed with petroleum ether and was eluted with the following solvents (table3.1).

Table 3.1: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50 mL each	Residue on evaporation	Melting point
Petroleum ether	1-4	oil	-
Petroleum ether: ethyl acetate (4:1)	5-8	nil	-
Petroleum ether: ethyl acetate (3:2)	9-14	solid	222^0-224^0C

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

1.3: Examination and characterization of Fractions: Same solids obtained from the reactions (Table-3.1) were combined (4 g) and crystallized from a mixture of chloroform-methanol to afford colorless needle shaped crystals of methylbetulinate of m.p. 223^0-224^0C, $[\alpha]_D$ +5.0. The compound was found identical with an authentic sample (mixed m.p, co-IR and co-tlc *etc.*). [Lit.70 m.p 224^0C-225^0C, $[\alpha]_D$ +5.0]



(72)
Methyl betulinate

Analysis report	%C	%H
Present compound	78.79	10.52
Calculated for $C_{30}H_{50}O_3$	79.10	10.71

IR: ν_{Nujol}^{max} 3540 (-OH),1730 (-$COOCH_3$) 1660, 890 cm^{-1} (=CH_2)

1.4. Preparation of Bromo derivatives of Betulinic acid:

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

1.4.1. Hydrogenation of Methyl betulinate:preparation of Methyl dihydro-betulinate

Methyl betulinate (4 g) dissolved in ethyl acetate (250 mL) was shaken in an atmosphere of hydrogen in presence of palladized charcoal (10%) catalysts (50 mg) for three hours until absorption of hydrogen ceased. Ethyl acetate was removed by distillation after filtering off the catalysts. The solution was diluted with water whereby a white solid (3.5 g) separated out which was collected by filtration. Crystallization from a mixture of chloroform-methanol furnished colorless needles of a compound, m.p 236^0-238^{0}C, $[\alpha]_D$ -17.0. This compound was found to be identical with an authentic sample of methyl dihydro betulinate (mixed m.p, co-IR and co-tlc).

IR: ν_{Nujol}^{max} 3540 (-OH), 1705 cm^{-1} (-CO_2Me)

1.4.2. Jone's oxidation of lupanol: Preparation of Methyldihydrobetulinate.

To a solution of methyl dihydro betulinate (3.3 g) in pure acetone (500 mL), Jone's reagent was added drop wise and with continuous shaking until a faint orange colour persisted. The mixture was kept at room temperature for 1 hour, then diluted with water and extracted with ether. The ether layer was washed thoroughly with water, dried by using anhydrous Na_2SO_4 and the solvent was evaporated. The residue (2.9 g) dissolved in minimum volume of petroleum ether was chromatographed over a column of silica gel (20 g). The chromatogram was developed with petroleum-ether and then eluted with the following solvents (Table3.2).

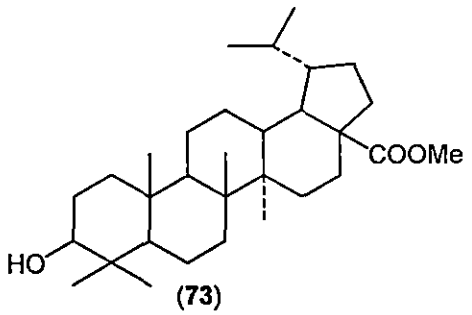
Table 3.2: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting' point
Petroleum ether	1-4	oil	-
Petroleum ether: ethyl acetate (4:1)	5-20	solid	197^0-199^{0}C

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

Fractions 5-20 (Table-3.2) were combined and crystallized from methanol furnished some needle shaped crystals, m.p. 192^{0}C-193^{0}C, $[\alpha]_D$ +18°, identical with an authentic sample of methyl dihydro betulonate (mixed m.p, co-IR and co-tlc).

[Lit 71 m.p.194^{0}C, $[\alpha]_D$ + 18.4°]



(73)

Methyl dihydrobetulinate

Analysis report	%C	%H
Present compound	79.32	10.86
Calculated for $C_{31}H_{50}O_3$	79.10	10.71

IR: ν_{Nujol}^{max} 1730 (-CO_2Me), 1708 cm^{-1} (C=O)

1.4.3: Treatment of Methyl dihydrobetulonate with N-bromo succinimide: formation of 2,2-dibromo methyl dihydrobetulonate (Compound F) and 2α-bromo methyl dihydrobetulonate (Compound G):

A solution of methyl dihydrobetulonate (2.5 g) in chloroform (125 mL) was mixed with dimethyl sulphoxide (65 mL) N-bromosuccinimide (3.0 g) and added 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. The residue (2.3 g) obtained after usual work up showed two spots on TLC plate, thus existence of at least two compounds were indicative. The residue (2.3 g) was chromatographed over a column of silica gel (20 g). The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 3.3).

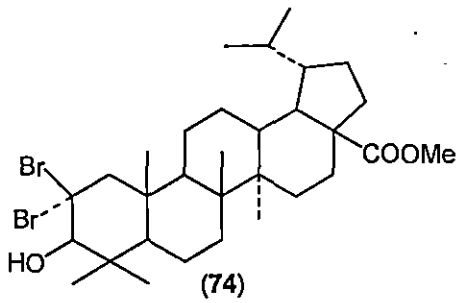
Table 3.3: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1-3	nil	-
Petroleum ether: ethyl acetate (4:1)	4-9	White solid	158^0-159^0C
Petroleum ether :ethyl acetate (4:1)	10	nil	-
Petroleum ether: ethyl acetate (4:1)	11-18	White solid	120^0-122^0C

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

1.4.4: Examination of fraction 4-9 (Table 3.3): Isolation of 2,2-dibromomethyl dihydrobetulonate (compound F):

The fractions 4-9 (Table 3.3) showed homogeneity on TLC plate were combined (0.9 g) and crystallized from of mixture of chloroform-methanol afforded 2,2-dibromomethylcdihydrobetulonate same as compound 'F', m.p 160^0-162^0C. It gave positive Beilstein test for halogens.



(74)

2,2-Dibromomethyl dihydrobetulinate

Analysis report	%C	%H
Present compound	58.82	12.72
Calculated for $C_{31}H_{48}O_3Br_2$	59.05	12.90

UV: 219 nm (e = 7879)

IR: ν_{Nujol}^{max} 1725 cm^{-1} (-CO_2Me), 1705 cm^{-1} (C=O)

1H NMR ($CDCl_3$): 0.76 to 1.22 ppm for 7 methyls,
3.11 and 3.63 (2d, 1H,1-CH2, J = 16Hz)
3.65 (s, 3H, -$COOCH_3$) ppm

MASS: m/z at 628, 626, 624 (M+), 571, 569, 567, [M-$COOCH_3$], 550, 548, 547[MHBr]+, 533, 531, 525, 523, 468, 470, 471, 453, 412, 411, 410, 409, 283, 285, 274, 231, 205, 203, 177 (base peak)

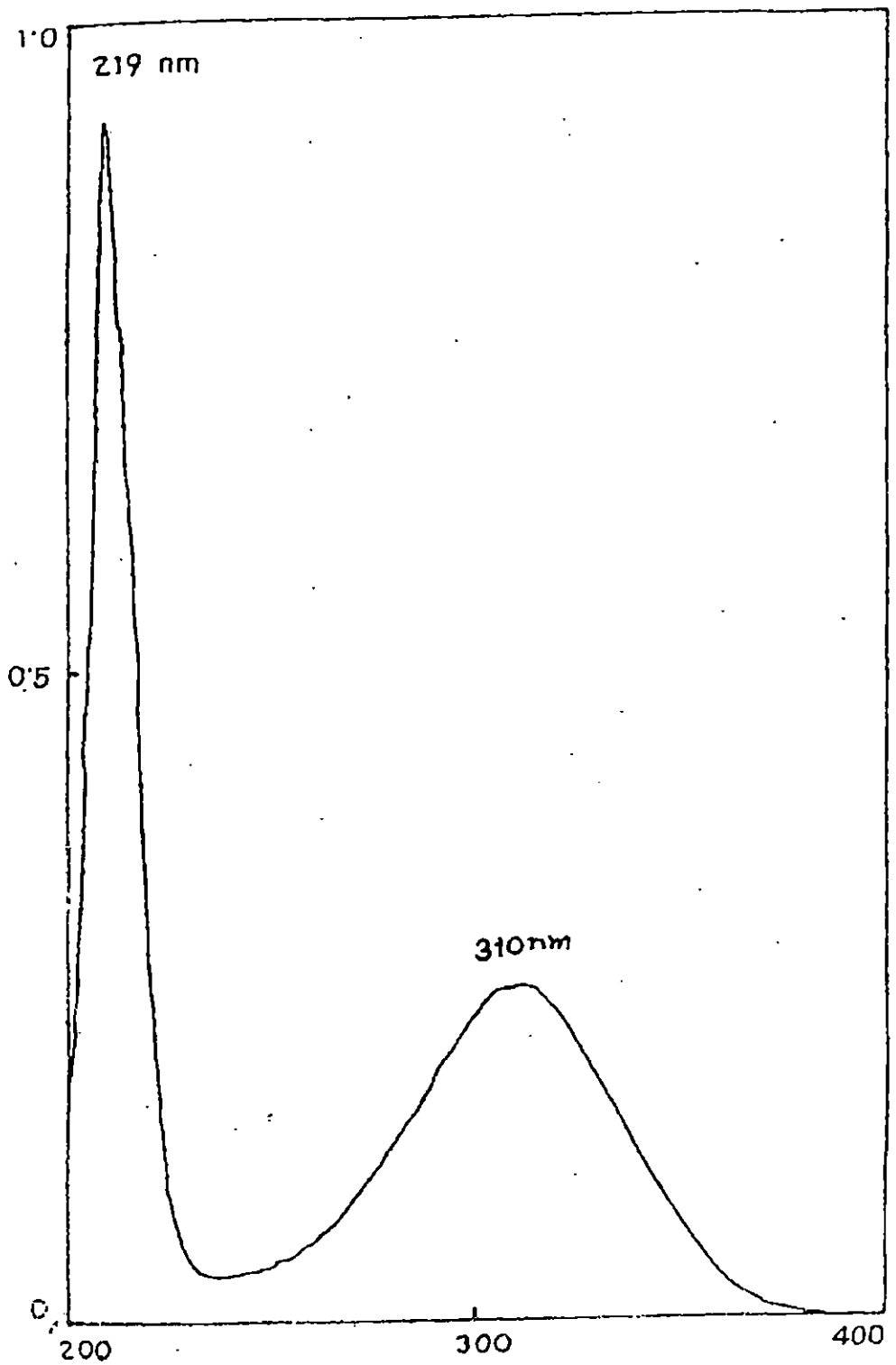


Fig. 23: UV Spectrum of 2,2– .bromo methyl dihydro betulonate (Compound F)

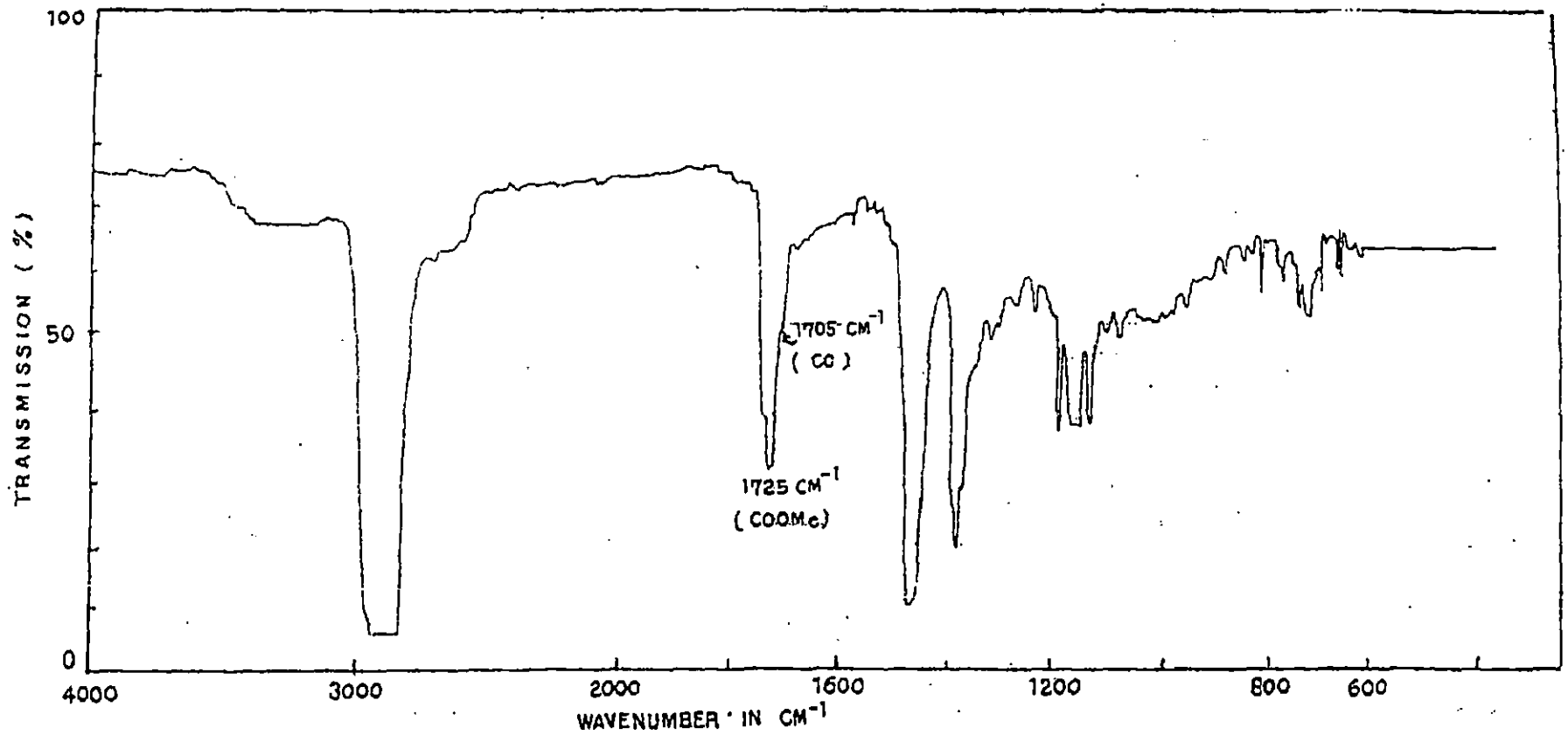


Fig. 24: IR Spectrum of 2,2-dibromo methyl dihydro betulonate (Compound F)

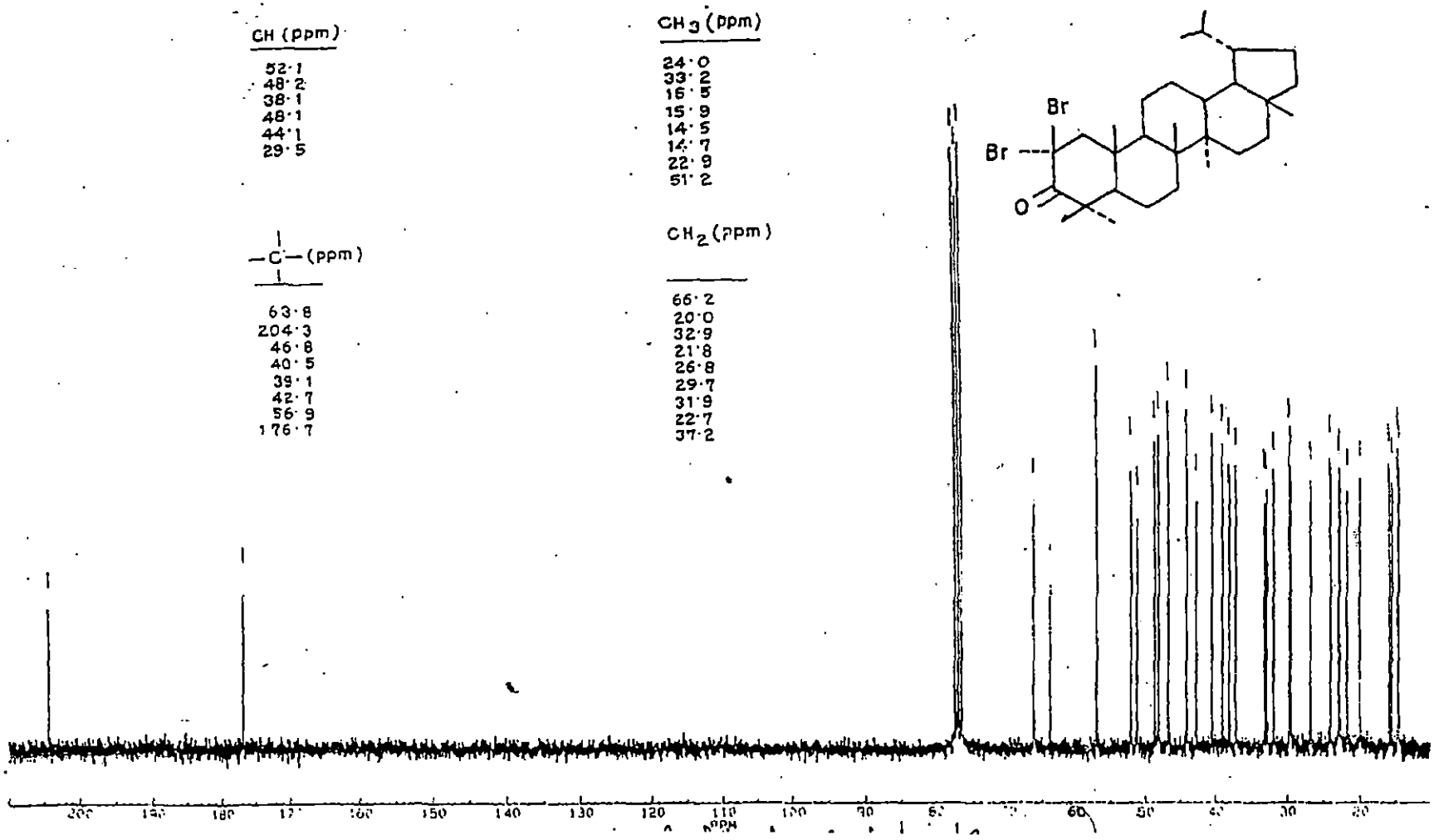
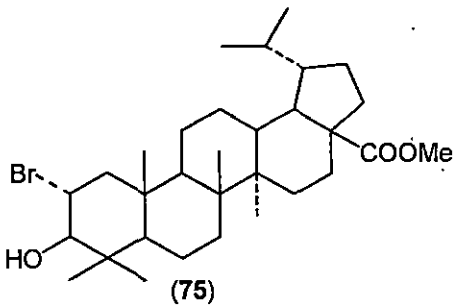


Fig. 25: ^{1}H NMR 2,2-dibromomethyldihydro betulonate (Compound F)

1.4.5: Examination of fractions 11-18 (Table 3.3): Isolation of 2αbromo methyl dihydro betulonate (compound G):

The fractions 11-18 (Table3.3) were combined (1.2 g) and crystallized with chloroform-methanol mixture to afford crystals of 2α-bromo methyldihydrobetulonate same as compound G, m.p 125^0-127^0C responded to belistein test for halogen and was identified as 2α-bromo methyl dihydro betulonate.



(75)

2α–Bromomethyl dihydrobetulinate

Analysis report	%C	%H
Present compound	67.21	13.18
Calculated for $C_{31}H_{49}O_3Br$	67.64	13.77

IR: : ν_{nujol}^{max} 1725 (-COOMe), 1705 cm^{-1}(C=O)

1H NMR ($CDCl_3$): 0.94, 0.97, 1.09,1.13, 1.20
(4s, 12H, 4t-CH_3)
0.77 and 0.87 (2d, 6H, 2s-CH_3, 1-CH J = 7 Hz)
2.65 (dd, 1H, 1-CH, J = 12 Hz and 6 Hz)
2.67 (t, 1H, 1C-H, J = 12 Hz)
5.06 (dd, 1H, 2-CH, J = 12 and 6 Hz) ppm

MASS: m/z at 550, 548(M+) (1:1)+, 491, 489, [M-COOCH3], 471, 470, 469, [M-Br]; 412, 411, (100%), 410, 395, 275, 260, 250, 205, 191, 177, 174, 119 (base peak) .

1.4.6. Dehydro bromination of 2α-bromo dihydro methyl betulonate: Isolation of 28carbomethoxy lup-1(2)-en-3-one (compound-H)

Compound G (200 mg) was refluxed with distilled DMF (30 mL) and lithium bromide (300 mg) for six hours. The residue (16g) obtained after usual work up was dissolved in minimum volume of benzene and chromatographed over a column of silica gel (15 g). The column was developed with petroleum ether and eluted with the following solvents (Table 3.4).

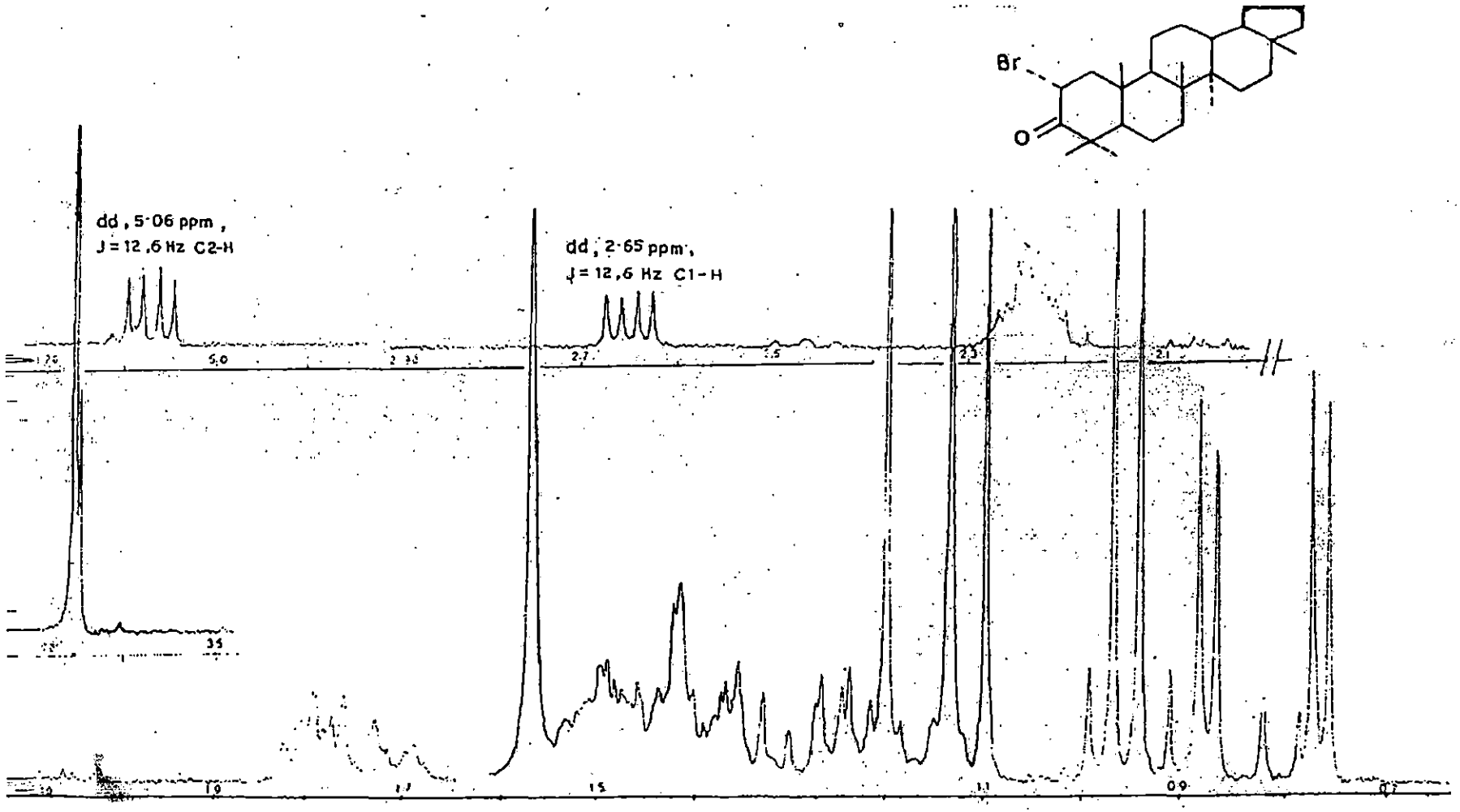


Fig. 26: ^{1}H NMR of 2α– bromo methyl dihydro betulonate (Compound G)

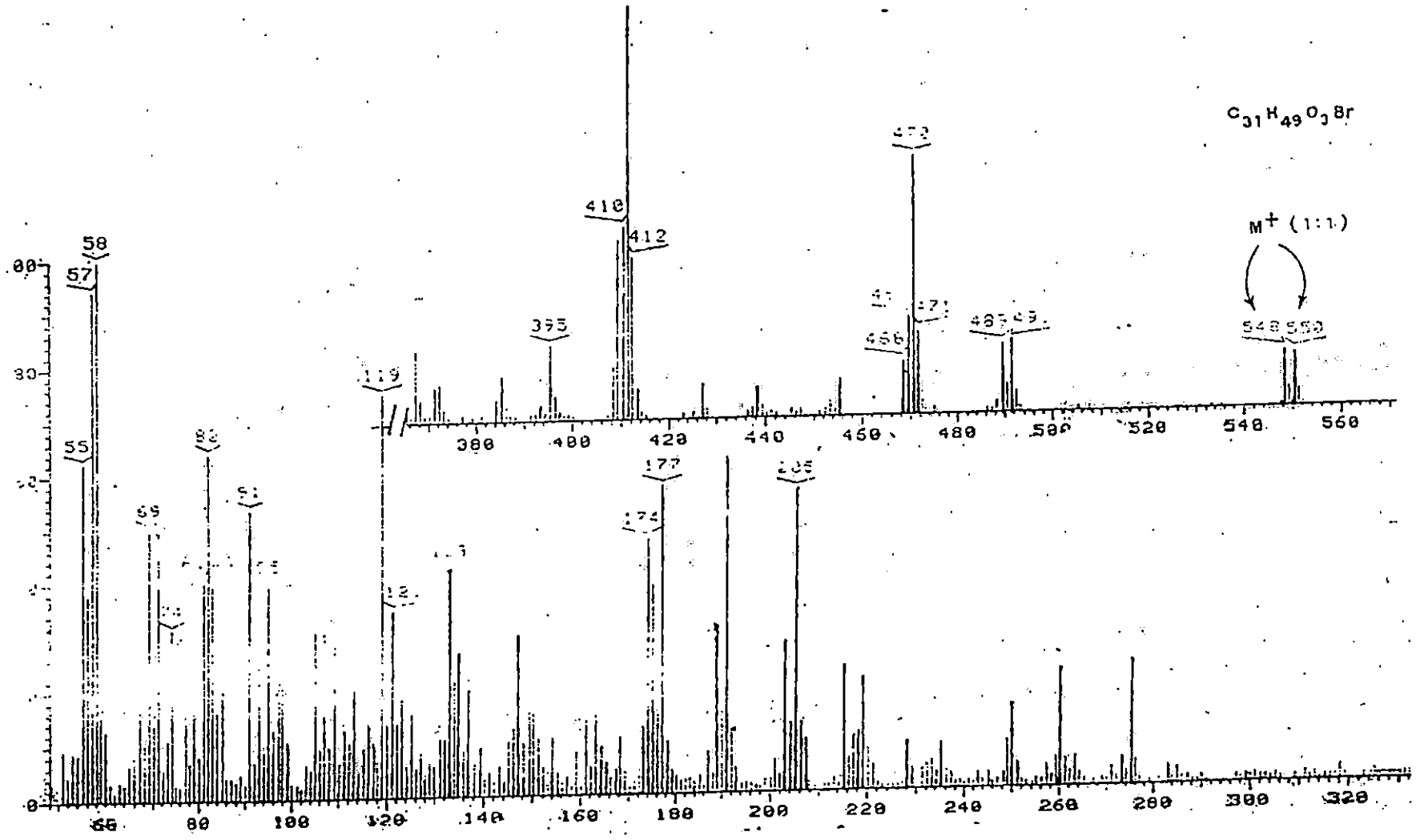


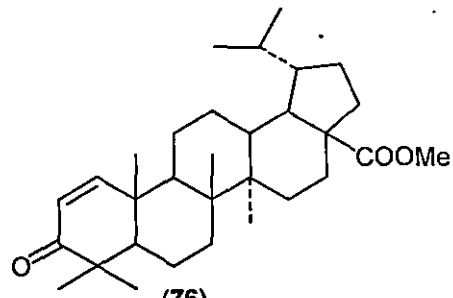
Fig. 27: Mass spectrum of 2α– bromo methyl dihydro betulonate (Compound G)

Table 3.4: Examination of different fractions obtained from column chromatographic separation

Eluent	Fractions 50ml each	Residue on evaporation	Melting point
Petroleum ether	1-3	nil	-
Petroleum ether :ethyl acetate(1:4)	4-14	nil	180^0-183^0C

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

Fractions 4-14 (Table 3.4) showed homogeneity on TLC plate were combined (0.15 g) and crystallized from a mixture of chloroform-methanol. The crystallization furnished colorless needle shaped crystals of compound H, m.p 186^0-187^0C and showed negative test for halogen and was identified as 28-carbomethoxy-lup-1(2)-ene-3-one same as compound H.



(76)
28-Carbomethoxy lup-1(2)-3-one

Analysis report	%C	%H
Present compound	79.28	10.24
Calculated for $C_{31}H_{48}O_3$	79.49	10.26

Beilstein test: Positive

UV: λ_{MeOH}^{max} 228 nm (e = 18,000)
IR: : ν_{Nujol}^{max} 1735 cm^{-1} (-COOMe), 1670 cm^{-1} (-C=C-C-)

1H NMR ($CDCl_3$): 0.96, 1.00, 1.07, 1.09, 1.13, (5s,15H 5 t-CH_3), 0.78 and 0.88 (2d,6H,2 S-CH_3, J= 8 Hz), 3.86 (s, 3H, -COO-CH_3) 5.81 and 7.11 (2d, 2H, H-C=C-H, J = 10 Hz) ppm

MASS: m/z at 468 $[M]^+$ 408 [-CH_3COOH]+, 393, 275, 271, 215, 150, 137, 121, 93, 69 (Base peak).

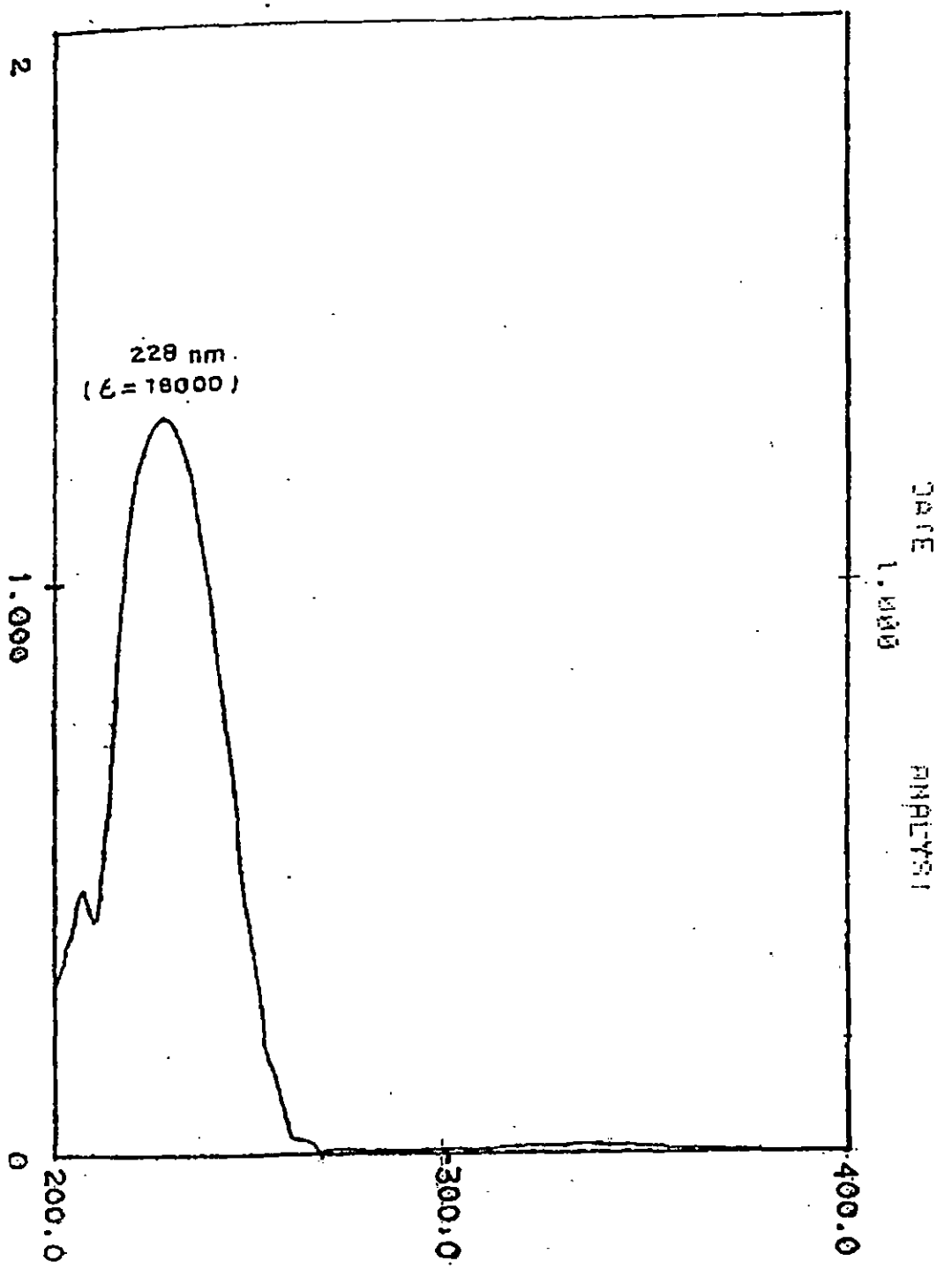


Fig. 28: UV Spectrum of 28-carbomethoxy lup-1-(2)-3-one (Compound H)

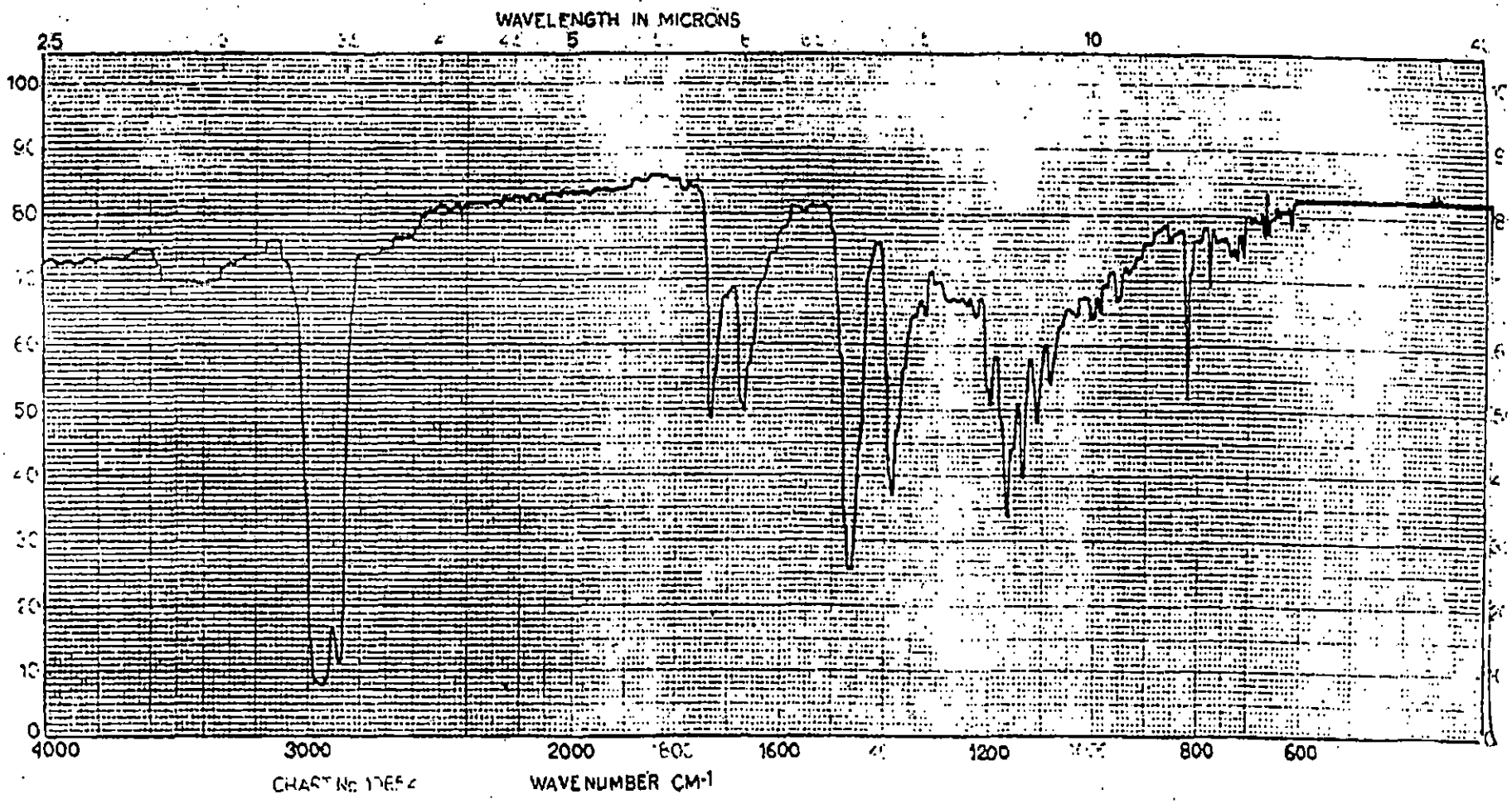


Fig. 29: IR Spectrum of 28-carbomethoxy lup-1-(2)-3-one (Compound H)

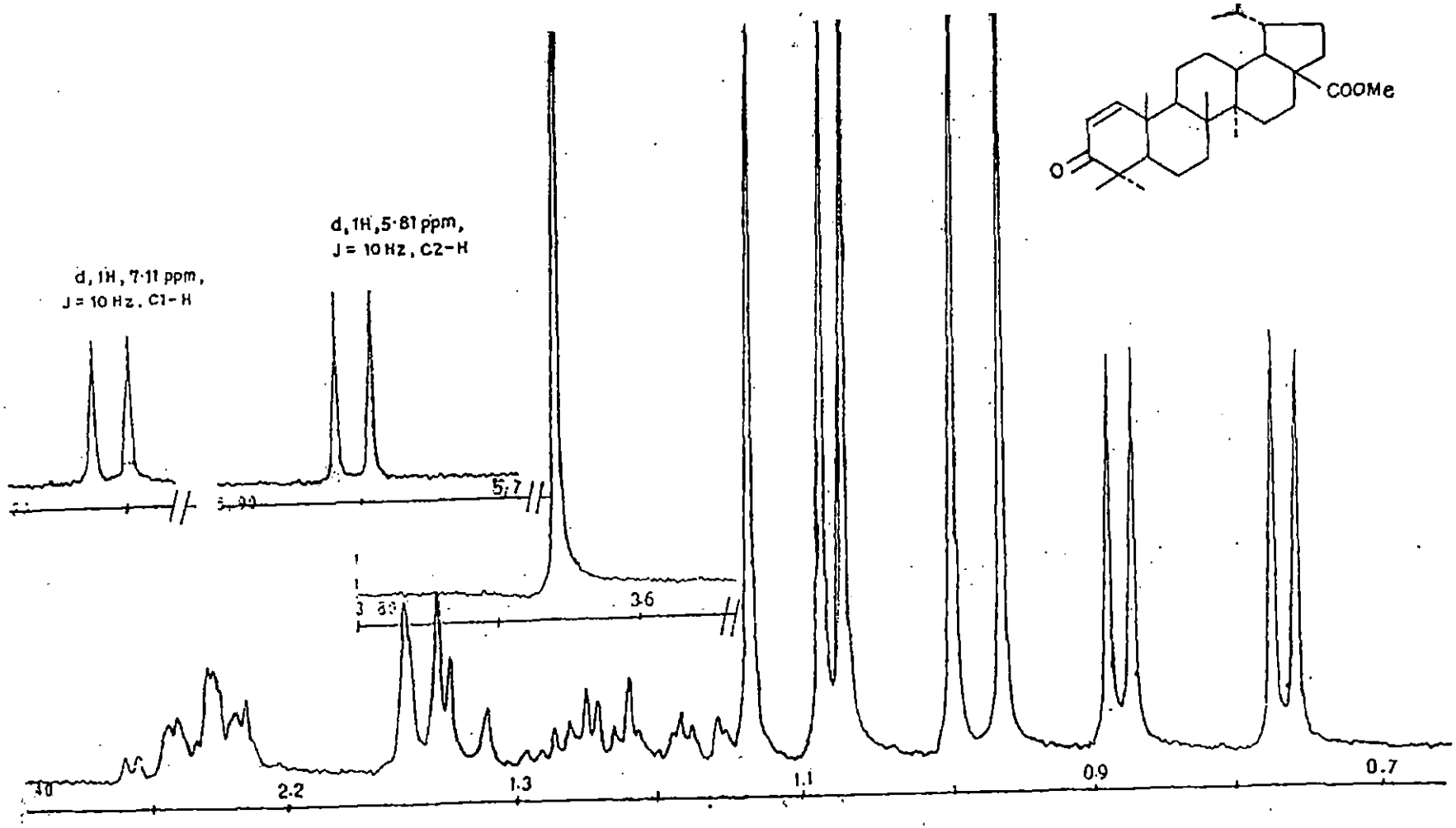


Fig. 30: ^{1}H IR Spectrum of 28-carbomethoxy lup-1-(2)-3-one (Compound H)

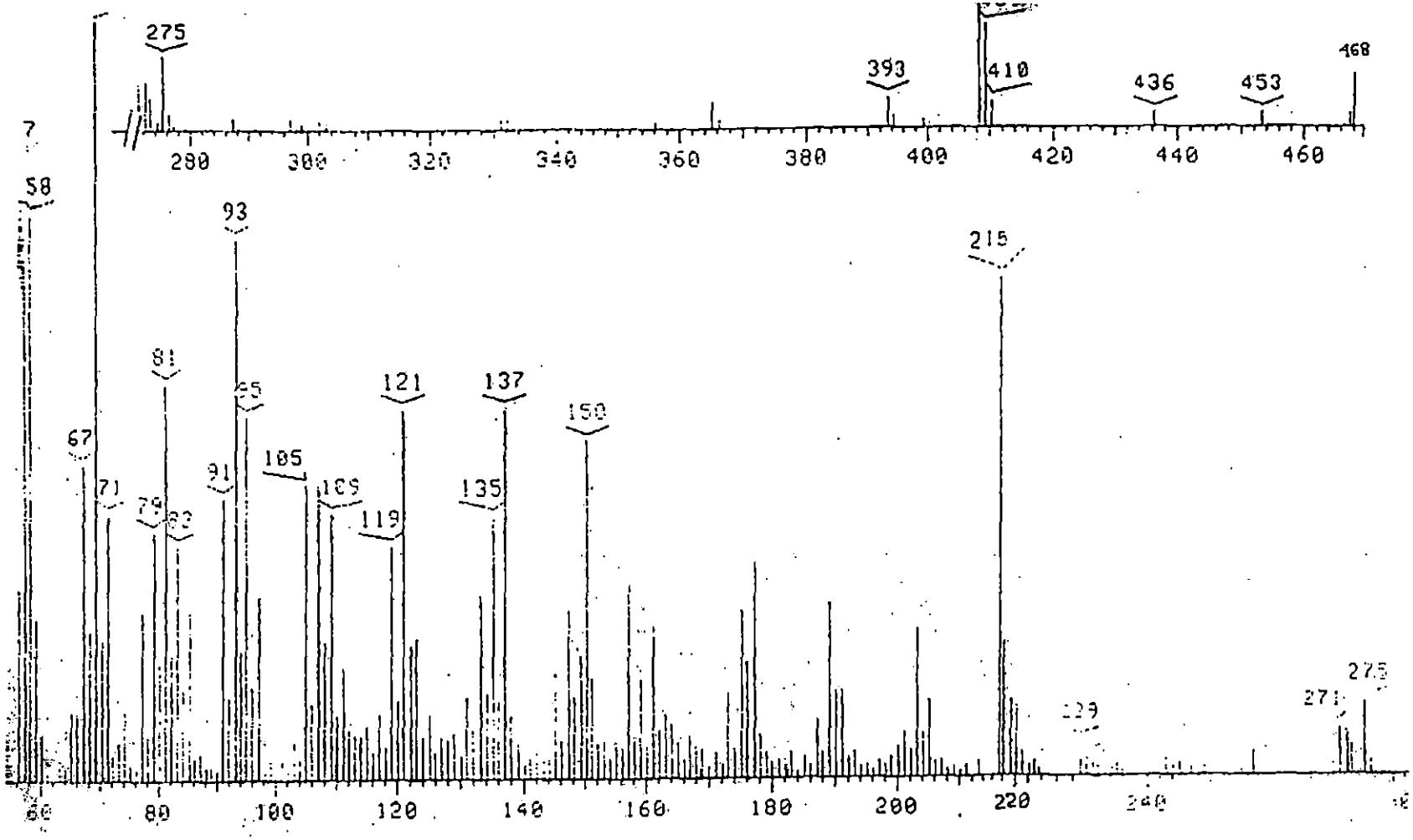
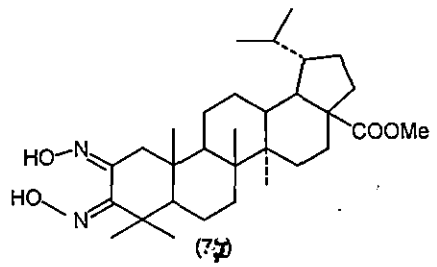


Fig. 31: Mass spectrum of 28-carbomethoxy lup-1-(2)-3-one (Compound H)

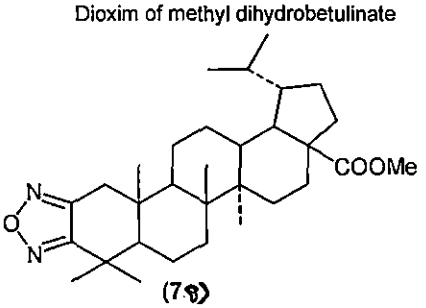
1.4.7. Cyclisation of the dioxime to 28-carbomethoxy lupane[2,3-C]-1′,2′,5′-Oxadiazole (compound - I)

28-Carbomethoxy-lupane[2,3-C]-1′,2′,5′-oxadiazole was prepared by cyclization of 28-carbomethoxy-2,3-dioximinolupane in dry DMF under microwave irradiation (100W, 100^{0}C) for 10 minutes. The residue obtained after usual work up was purified by repeated crystallization by chloroform-methanol mixture and designated as compound 'J', analyzed for $C_{31}H_{48}O_4N_2$ which was corroborated by mass spectral analysis that showed the molecular ion at m/z 496. The other ions appeared at m/z 496.72 [M-CH3], 496 (BASE PEAK), 497, 367, 271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81, 55. IR spectrum of the compound showed peaks at 1620 cm^{-1} (-C=N-O) and 890 cm^{-1}, 1720 cm^{-1} (-COOMe) for heterocyclic ring. It showed UV absorption maximum at 223 nm (ε = 5169) for disubstituted furazine derivative. The ^{1}HNMR were 0.77 (d, 3H, J = 7Hz), 0.78, 0.86 (d, 3H, J = 7Hz), 0.97, 1.10. It showed the presence of six tertiary methyls. A pair of one-proton doublets each at 2.1 (J = 16 Hz) ppm and 3.15 (J = 16 Hz) ppm may be due to the germinal coupling of the C-1 proton which were adjacent to the furazine ring. Thus from spectral analysis the structure of the compound 14 has been established as 28-carbomethoxy-lupan- [2,3-C]- 1′,2′,5′-oxadiazole (compound J).



(75)

Dioxim of methyl dihydrobetulinate



(76)

28-Carbomethoxy lupan [2,3-C]-1′,2′,5′-oxadiazole

Section 2: Antimicrobial activity

2.1: Materials and Methods:

Details of the materials and methods have already been mentioned in chapter II section 2.1

2.2. Results:

Compound F, chemical name 2,2-dibromo methyl dihydrobetulonate was extracted from the bark of *Bischofia javanica*, belongs to the family Euphorbiaceae [details of extraction procedure was given in chapter-III, section 1.1]. Pharmacological research [5] on the bark of the plant suggested that the bark has usable antifungal and antimicrobial properties [28-30,33,34].

From the results presented in table-3.5 it was found that 2,2-dibromo methyldihydrobetulonate (Compound-F) was highly effective in controlling the spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*. Spore germination of all the fungal pathogens was reduced to a significant level by all the five concentrations (100,200,300,400 & 500ppm) of the compound tested.

Table3.5: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 2,2-dibromo methyldihydrobetulonate (Compound-F) (when control raised to 100).

Fungal organism	Concentrations of compound(ppm)	Range of germtube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-52	13	87±2.00
	200	08-44	02	98±1.88
	300	08-32	01	99±1.69
	400	-	-	100±1.00
	500	-	-	100±1.00
Fusarium equiseti	100	08-16	25	75±1.50
	200	04-12	15	85±1.75
	300	04-12	07	93±1.52
	400	04-08	04	96±1.88
	500	04-08	nil	100±1.00
Curvularia eragrostidis	100	40-112	23	77±1.20
	200	32-72	12	88(S=±1.34)
	300	28-56	10	90(S=±1.99)
	400	20-48	08	92(S=±1.58)
	500	16-32	06	94(S=±1.35)

*Data after ± indicates standard error value

Antifungal and antibacterial activity of the compound-F was also tested following disc diffusion method. Results of the test have been presented in the table-3.6, table-3.7 & plate-7. Compound-F did not show any antifungal activity against *C. eragrostidis* but it could check growth of the two fungi (*C.gloeosporioides* and *F.equiseti*) as evident from the results presented in table-3.6. The diameter of inhibition zones ranged between 1.2 to 2.5cms. The results of antibacterial activity of Compound-F have been presented in table-3.7. From the results it was evident that growth of *R. solanacearum* could be inhibited at 200ppm concentration and above. Growth of all other bacteria (*Xanthomonas sp, P.syringae* and *E.carotovora*) tested was controlled even at concentration of 100 ppm. Largest diameter (2.8cms) of antifungal zone was observed at 500ppm concentration of the compound against *R. solanacearum*.

Table 3.6: Antifungal Activity of 2,2-dibromo methyldihydrobetulonate (compound'F')

Fungal organism	Concentrations of compounds(ppm)	Diameter of inhibition zone(cm)*
Colletotrichum gloeosporioides	100	1.2
	200	1.5
	300	1.9
	400	2.2
	500	2.5
Fusarium equiseti	100	1.6
	200	1.8
	300	1.9
	400	2.0
	500	2.3
Curvularia eragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

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

Table 3.7: Antibacterial activity of 2,2dibromomethyldihydrobetulonate (Compound'F')

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.4
	200	2.4
	300	2.5
	400	2.7
	500	2.8
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

In the present study, 2α-Bromo methyldihydrobetulonate (Compound-G) was used as seventh compound for control of fungal and bacterial pathogens. Initially, the spore germination bioassay was performed. The results of the spore germination bioassay have been presented in Table 3.8. More than 90% reduction in spore germination was experienced at 500ppm concentration of the compound-G against the three fungi (*Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis*). Cent percent spores of *Fusarium equseti* could not germinate in presence of the compound G even at 100 ppm concentration.

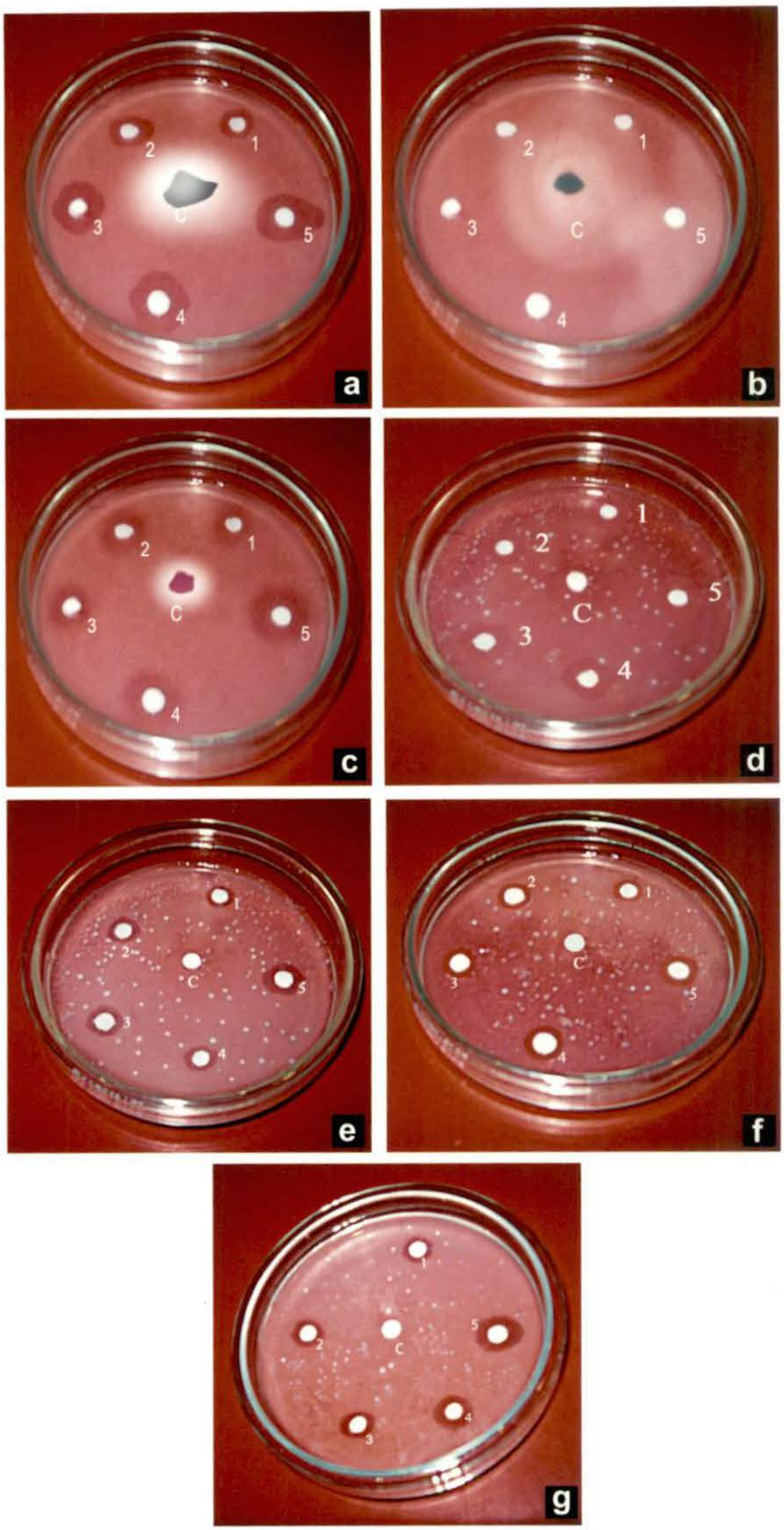


Plate 6: Disc diffusion test for anti microbial activity of compound F:(2,2-dibromomethyl dihydrobetulonate); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

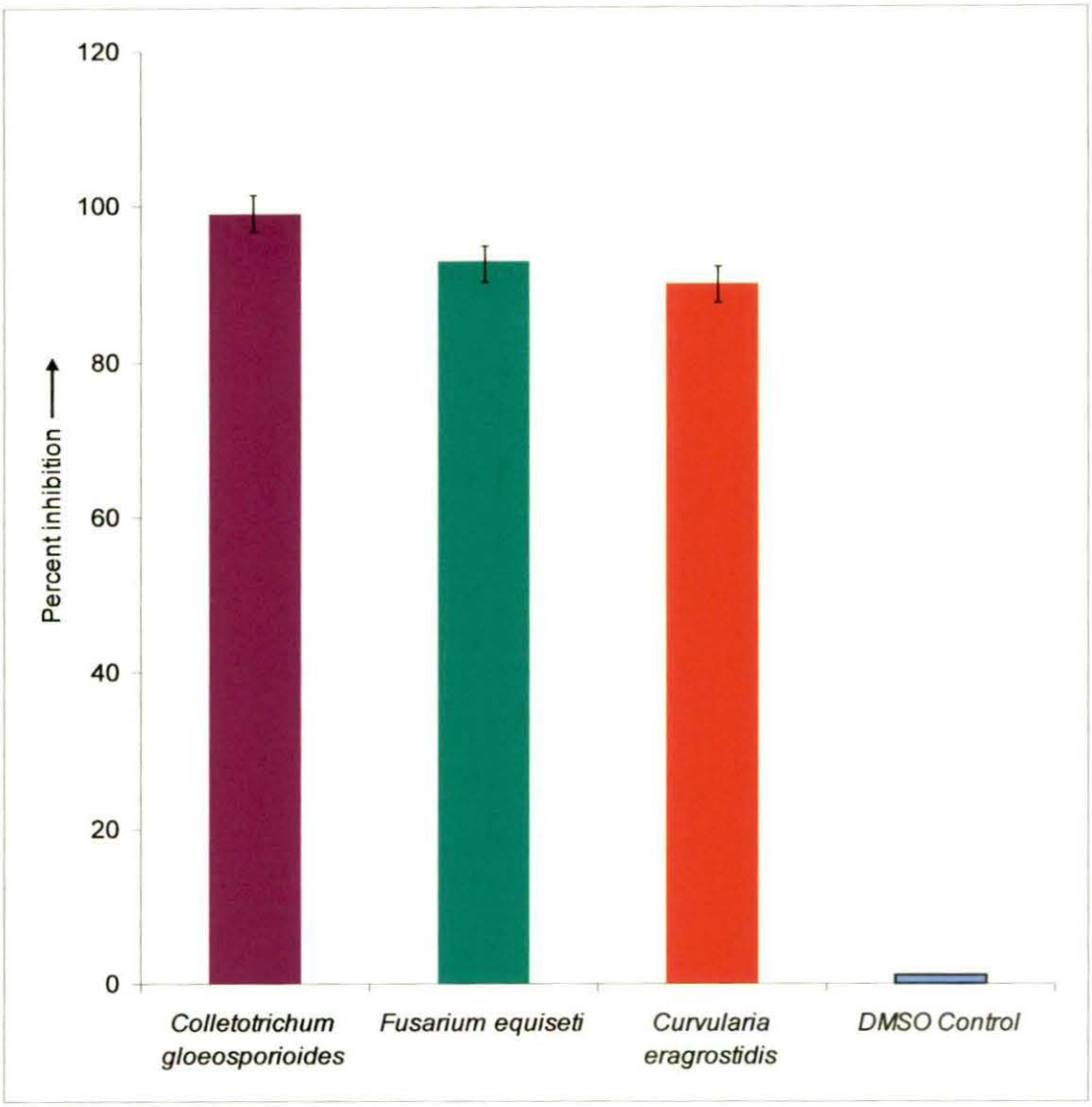


Fig. 33: Percent inhibition of spore germination at 300 ppm concentration of compound F (2,2-dibromo methyldihydrobetulonate).

Table 3.8: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 2α-Bromomethyldihydrobetulonate (Compound 'G') (when control raised to 100)

Fungal organism	Concentrations of compound(ppm)	Range of germ tube Length(micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-52	26	74±1.30
	200	08-40	11	89±1.94
	300	08-32	10	90±1.66
	400	04-32	07	93±1.78
	500	04-20	04	96±1.90
Fusarium equiseti	100	08-20	12	88±1.80
	200	08-16	07	93±1.60
	300	04-12	04	96±1.68
	400	04-12	01	99±1.00
	500	-	-	100±1.00
Curvularia eragrostidis	100	40-108	21	79±1.40
	200	32-80	17	83±1.95
	300	28-56	13	87±1.84
	400	24-48	08	92±1.36
	500	20-40	07	93±2.00

*Data after ± indicates standard error value

Results presented in table-3.9 and plate-8 represents the antifungal activity of Compound-G (through disc diffusion bioassay) at five different concentrations. From the results of antifungal activity of compound–G (2α-Bromo methyldihydrobetulonate), it was evident that the compound at all the five different concentrations could check the growth of two pathogens (*C.gloeosporioides* and *F.equiseti*) but it could not control spore germination of *C. eragrostidis.*

In table 3.10, the results of antibacterial activity of compound-G (2α-bromomethyldihydrobetulonate) have been presented. Compound-G was effective from 100ppm concentration against the bacteria tested. Diameter of inhibition zones ranged between 0.7 to 2.1 cm. Best control (diameter of inhibition zone 2.1cm) of growth of the bacterium (*P.syringae*) was observed at 500ppm concentration.

Table 3.9: Antifungal activity of 2α-Bromo methyldihydrobetulonate (Compound 'G')

Fungal organism	Concentrations ofcompounds(ppm)	Diameter of inhibition zone(cm)*
Colletotrichum gloeosporioides	100	0.8
	200	0.9
	300	1.0
	400	1.2
	500	1.4
Fusarium equiseti	100	1.4
	200	1.7
	300	1.9
	400	2.0
	500	2.1
Curvularia eragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

Table3.10: Antibacterial activity of 2α-bromomethyldihydrobetulonate (compound 'G')

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.7
	200	1.0
	300	1.2
	400	1.4
	500	1.8
Xanthomonas sp	control	-
	100	0.5
	200	0.8
	300	1.0
	400	1.3
	500	1.5
Pseudomonas syringae	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.8
	500	2.1
Erwinia carotovora	Control	-
	100	0.7
	200	0.8
	300	1.0
	400	1.3
	500	1.8

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

Compound-H (28-carbomethoxylup-1(2)-en-3-one) was the eight compound tested for the antimicrobial activity against three fungal and four bacterial pathogens. Details of the extraction and synthesis of the compound have been mentioned in the section- 1of chapter-III. From the results presented in table 3.11and plate-9 it was found that 100ppm concentration of the compound could reduce spore germination up to 73%, 77% and 87% respectively in case of *Fusarium equiseti, Colletotrichum gloeosporioides* and *Curvularia eragrostidis.* More than 95% inhibition of spore germination was experienced when 500 ppm concentration of the compound was used against the three test pathogens.

Table 3.11: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 28-carbomethoxylup-1(2)-en-3-one (Compound 'H') (when control raised to 100)

Fungal organism	Concentrations of compound (ppm)	Range of germ tube length(micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	23	77±1.13
	200	12-40	05	95±1.80
	300	08-36	03	97±1.65
	400	08-24	02	98±1.88
	500	04-20	01	99±1.66
Fusarium equiseti	100	12-20	27	73±1.70
	200	08-20	12	88±1.38
	300	08-20	10	90±1.55
	400	04-12	08	92±1.30
	500	04-08	05	95±1.45
Curvularia eragrostidis	100	36-100	13	87±1.87
	200	24-72	08	92±1.99
	300	28-56	07	93±1.32
	400	24-48	05	95±1.70
	500	20-40	04	96±1.80

*Data after ± indicates standard error value

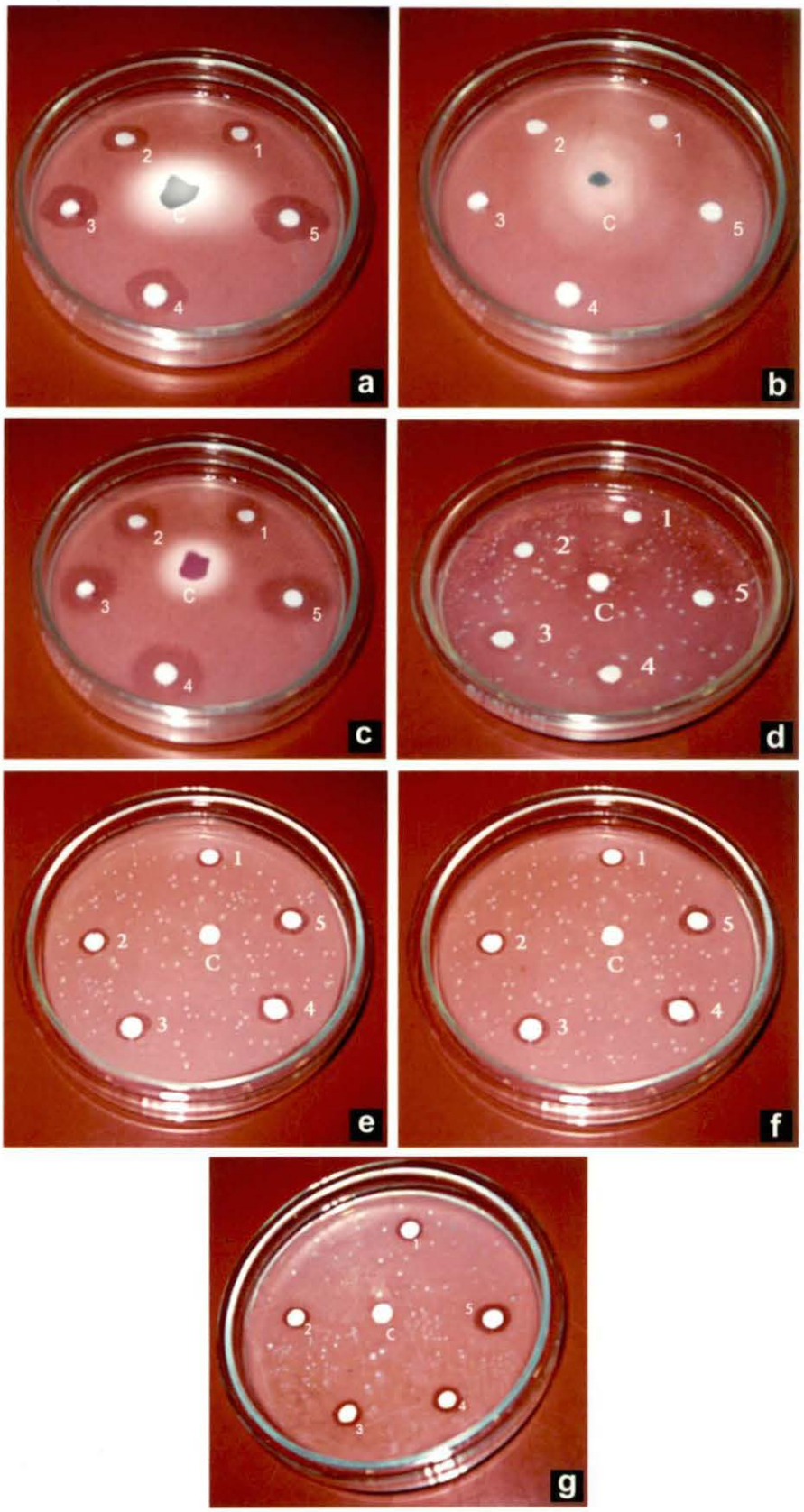


Plate 7: Disc diffusion test for anti microbial activity of compound G: (2α-bromo methyldihydrobetulonate); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

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

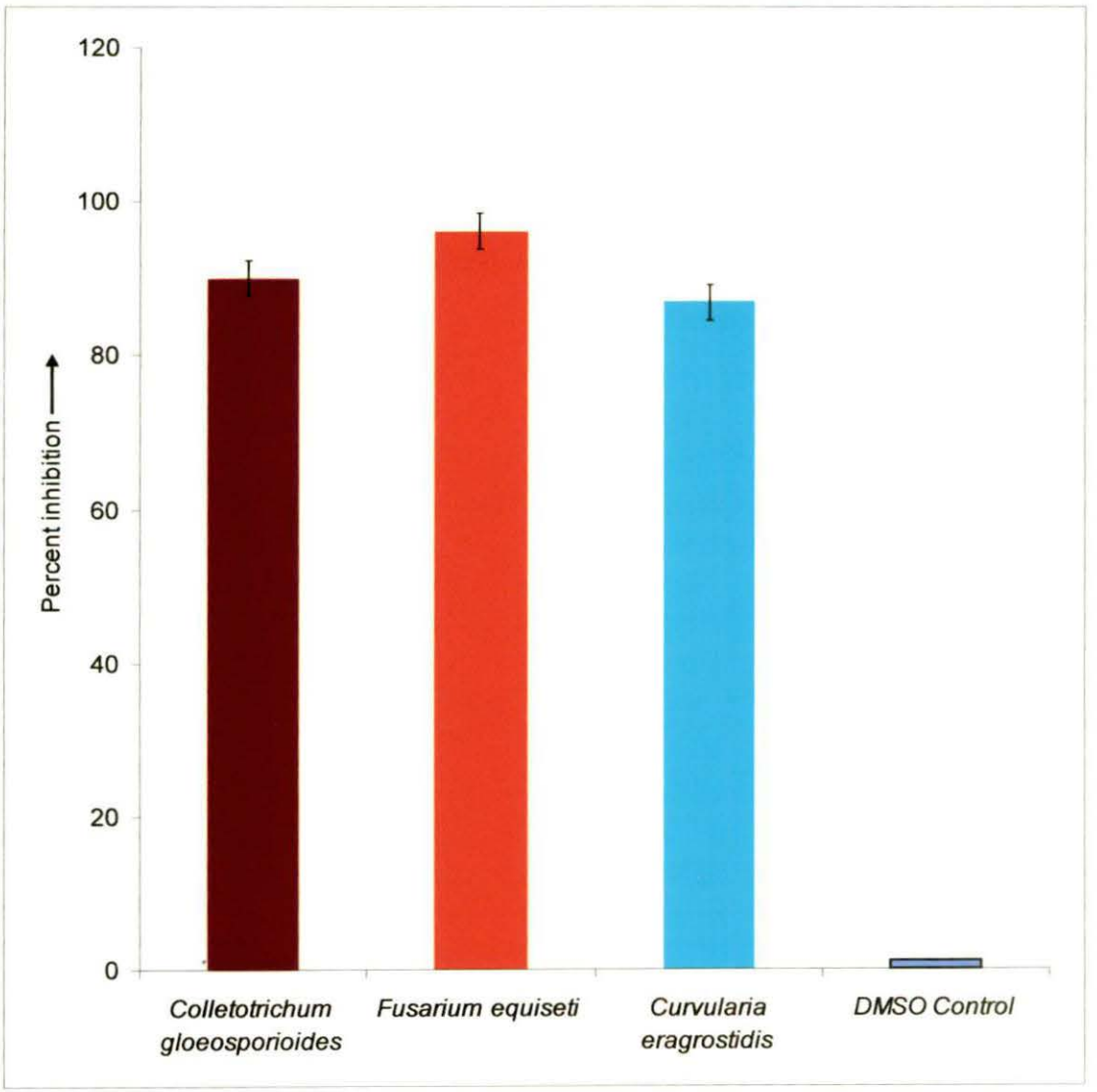


Fig. 34: Percent inhibition of spore germination at 300 ppm concentration of compound G (2α-bromomethyldihydrobetulonate).

Results of the disc diffusion method for evaluation of antimicrobial activity of Compound-H have been presented in table 3.12, 3.13 & plate-9. All the five concentrations of compound-H showed antifungal activity against *Colletotrichum gloeosporioides* and *Fusarium equiseti* but it could not inhibit the growth of *Curvularia eragrostidis* even at 500 ppm concentration. Thus the compound was found to be selectively antifungal.

In table 3.13 the results of antibacterial activity of 28-carbomethoxy lup-1(2)-en-3-one (compound H) was presented. Compound-H was effective in all the five concentrations tested. Largest antibacterial zone was found in case of *Pseudomonas syringae* when tested against 500ppm concentration of the compound. Growth of *R.solanacearum, Xanthomonas* sp and *E.carotovora* were also controlled by the compound. Best control was experienced at 500 ppm concentration of the compound.

Table 3.12: Antifungal activity of 28-carbomethoxylup-1(2)-en-3-one (Compound 'H')

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	1.4
Fusarium equiseti	100	1.3
	200	1.5
	300	1.6
	400	1.7
	500	1.9
Curvularia eragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

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

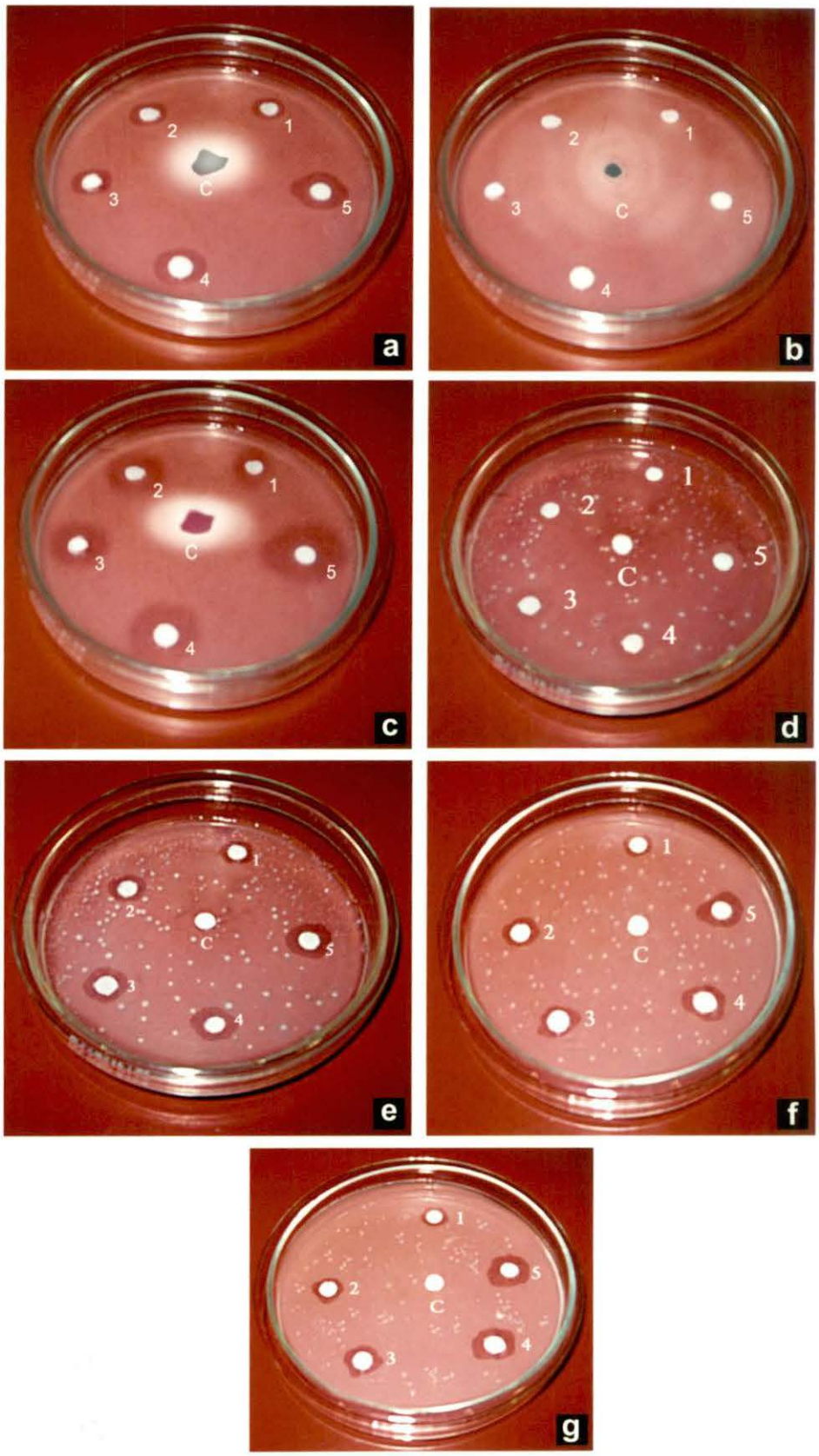


Plate 8: Disc diffusion test for anti microbial activity of compound H: (28-carbomethoxy lup-1(2)-en-3-one); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora* (g) *Xanthomonas* sp.

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

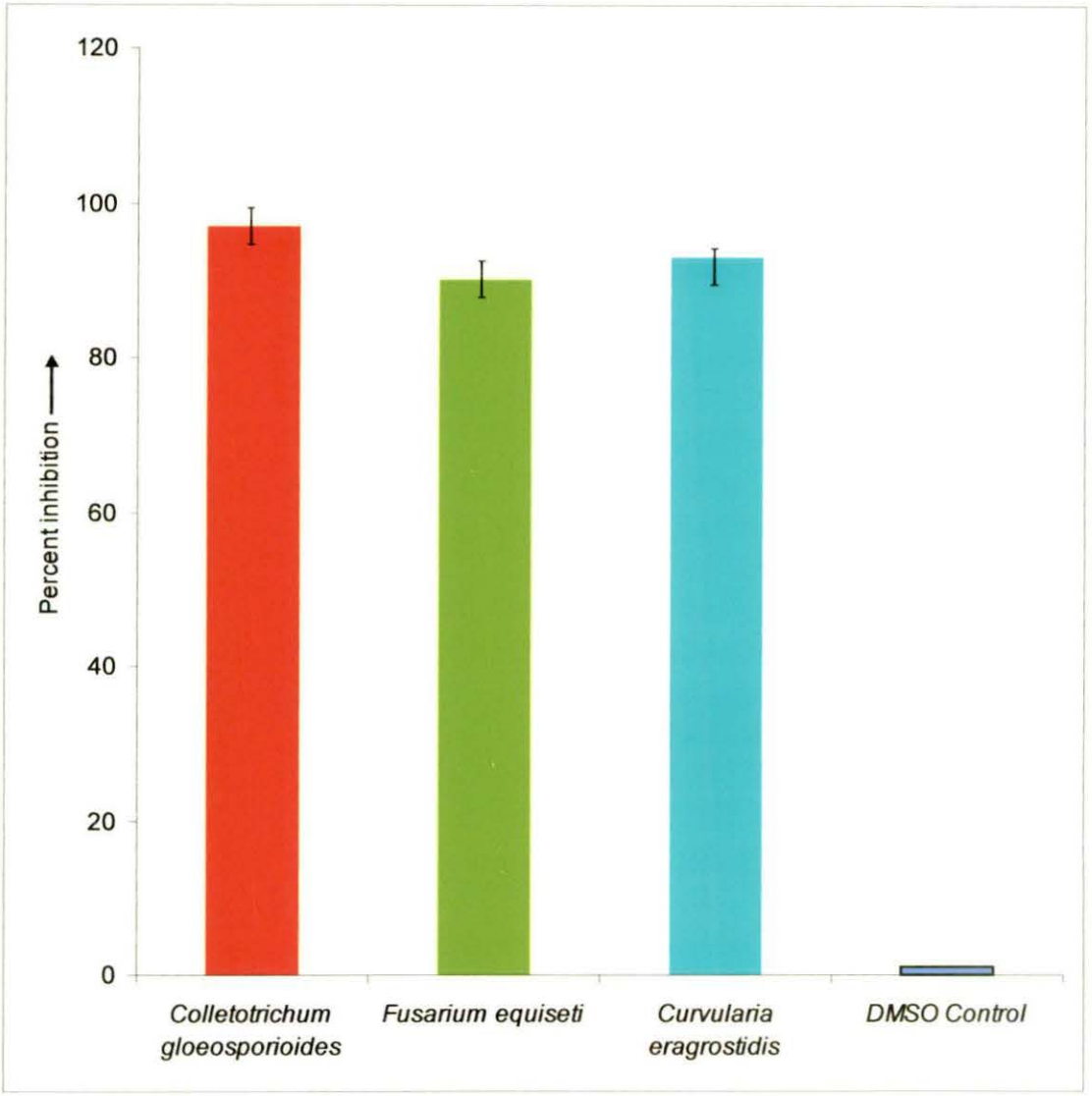


Fig. 35: Percent inhibition of spore germination at 300 ppm concentration of compound H (28-carbomethoxy lup-1(2)-en-3-one).

Table 3.13: Antibacterial activity of 28-carbomethoxy lup-1(2)-en-3-one (compound-H)

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.7
	200	1.0
	300	1.2
	400	1.4
	500	1.8
Xanthomonas sp	control	-
	100	0.5
	200	0.8
	300	1.0
	400	1.3
	500	1.5
Pseudomonas syringae	Control	-
	100	1.0
	200	1.2
	300	1.5
	400	1.8
	500	2.1
Erwinia carotovora	Control	-
	100	0.7
	200	0.8
	300	1.0
	400	1.3
	500	1.8

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

Compound-I (identical as 28-Carbomethoxy lupan[2,3-C]-1́,2́,5́-oxadiozole) was formed by cyclisation of 28-carbomethoxy-2,3-dioximinolupane. Details of the formation of the compound was mentioned in the section-1of chapter III. From the results presented in the table 3.14, it was found that the antifungal activity of 28-carbomethoxy lupan [2,3-C]-1́,2́,5́-oxadiozole (compound-I) was effective at higher concentrations. More than 90% inhibition of spore germination (of all the three fungi tested) was observed in case of 400ppm concentration of the compound.

Table3.14: Percent inhibition of spore germination of *Colletotrichum gloeosporioides, Fusarium equiseti* and *Curvularia eragrostidis* by 28-carbomethoxy lupane-[2,3-C]-1',2',5'oxadiozole(compound 'I') (when control raised to 100)

Fungal organism	Concentrations of compound (ppm)	Range of germ tube length (micrometer)	percent germination	Percent Inhibition*
Colletotrichum gloeosporioides	100	12-44	85	15 ±1.18
	200	12-40	32	68±1.50
	300	12-36	21	79±1.70
	400	08-32	05	95±1.85
	500	04-24	04	96±1.92
Fusarium equiseti	100	12-24	84	16±1.45
	200	10-20	18	82±1.60
	300	10-20	17	83±1.41
	400	08-16	10	90±1.86
	500	04-12	05	95±1.63
Curvularia eragrostidis	100	40-88	15	85±1.26
	200	32-80	11	89±1.54
	300	28-68	09	91±1.90
	400	24-56	07	93±1.52
	500	20-48	05	95±1.22

*Data after ± indicates standard error value

In disc diffusion test (presented in the table 3.15 & plate-10) compound–I could not check the mycelia growth of *Curvularia eragrostidis.* But, the other two fungi showed significant antifungal zone of inhibition against the compound. Diameter of inhibition zones was 2.1 and 2.2 cm in case of *Colletotrichum gloeosporioides* and *Fusarium equiseti* respectively at 500 ppm concentration of the compound-I.

In similar experiments (table 3.16 & plate-10) antibacterial activity was also evaluated against the compound. Compound-I was effective against all the four bacteria (*R.solanacerum, Xanthomonas sp, Pseudomonas syringae, E.carotovora*). The diameter of inhibition zones were between 0.6 to 1.5cm. Comparing the diameter of inhibition zones produced by other compounds against the bacteria, compound-I was not a strong antibacterial compound.

Table 3.15: Antifungal activity of 28-carbomethoxylupane[2,3-C]-1′,2′,5′-oxadiozole (compound 'I')

Fungal organism	Concentrations of compounds(ppm)	Diameter of inhibition zone(cm)*
Colletotrichum gloeosporioides	100	1.5
	200	1.6
	300	1.6
	400	1.9
	500	2.1
Fusarium equiseti	100	1.3
	200	1.7
	300	2.0
	400	2.0
	500	2.2
Curvularia eragrostidis	100	-
	200	-
	300	-
	400	-
	500	-

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

Table 3.16: Antibacterial activity of 28-carbomethoxy lupane[2,3-C]-1′,2′,5′-oxadiozole (compound-I)

Bacterial organism	Concentrations of compound (ppm)	Diameter of inhibition zone(cm)*
Ralstonia solanacearum	Control	-
	100	0.6
	200	0.8
	300	0.9
	400	1.0
	500	1.2
Xanthomonas sp	control	-
	100	0.4
	200	0.7
	300	1.1
	400	1.3
	500	1.5
Pseudomonas syringae	Control	-
	100	0.5
	200	0.6
	300	0.8
	400	0.9
	500	1.1
Erwinia carotovora	Control	-
	100	0.7
	200	0.9
	300	1.0
	400	1.1
	500	1.3

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

Phytotoxicity tests of Betulinic Acid and its derivatives:

Phytotoxicity of the four compounds were 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 maintained in the experimental garden of the Department of Botany with normal watering. Fifty such pots containing plants (each 10 cm in height) were kept in a net house. The plants were grouped into five sets and each set contained 10 plants each. Four compounds (at 100 ppm concentration) were sprayed in six sets separately and the fifth set was sprayed with sterile distilled water. The plants were observed up to seven days. No phytotoxicity was observed at 100ppm concentration of the compounds when compared with the control set which was sprayed with the sterile distilled water (table-3.17).

Table 3.17: Phytotoxicity of betulinic acid and its derivatives

Compounds	Concentrations (ppm)	Morphological & Physiological abnormalities abnormalities			
		1day	3day	5day	7day
F	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
G	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
H	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
I	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

Chapter III deals with an evergreen woody tree *Bischofia javanica* belongs to the family Euphorbiaceae. Betulinic acid was obtained through phytochemical extraction of *Bischofia javanica*. Then Methyl betulinate was isolated through estarification of betulinic acid and characterized. After that Methyl dihydrobetulinate was prepared by hydrogenation of methylbetulinate and was also characterized. Methyldihydrobetulonate was prepared by Jone's oxidation of betulinic acid and characterized. Treatment of methyldihydrobetulonate with N-bromosuccinimide, 2,2-dibromomethyldihydro-betulonate (compound F) and 2α-bromomethyldihydrobetulonate (compound G) were formed and characterization of these compounds were done. 28-carbomethoxy-lup (2)-en-3-one, compound H was prepared by dehydrobromination of 2α-bromodihydromethylbetulonate and characterized. Another derivative of betulinic acid named 28-carbomethoxy Lupane-[2,3-C]-1′,2′,5′- oxadiozole, called compound-I was prepared by cyclisation of 28-carbomethoxy 2,3-dioximolupane in dry DMF under microwave irradiation (100w,100^{0}C) for 10 min.

Compound-F (2,2-dibromo methyldihydrobetulonate) significantly controlled (100% inhibition) spore germination of *Colletotrichum gloeosporioides* and *Fusarium equiseti* at highest concentration (500ppm) tested. *Curvularia eragrostidis* was also controlled (inhibition was 94% at 500ppm concentration). From the results of disc diffusion test, it was evident that vegetative growth of *Curvularia eragrostidis* could not be controlled by compound-F. But it could control the vegetative growth of *Colletotrichum gloeosporioides* and *Fusarium equiseti* significantly (showed 2.5cm and 2.3cm diameter of inhibition zones respectively). Three bacteria (*Xanthomonas sp, Pseudomonas syringae* and *Erwinia carotovora*), tested against compound-F showed inhibition of growth. *R. solanacearum* could not be controlled at 100ppm concentration but it could be controlled significantly at 200ppm concentration.The inhibition zone diameter was found to be 2.4cm. However, the other three bacteria (*Xanthomonas sp, Pseudomonas syringae* and *Erwinia caratovora*) could be controlled at lowest concentration (100ppm) of the compound.

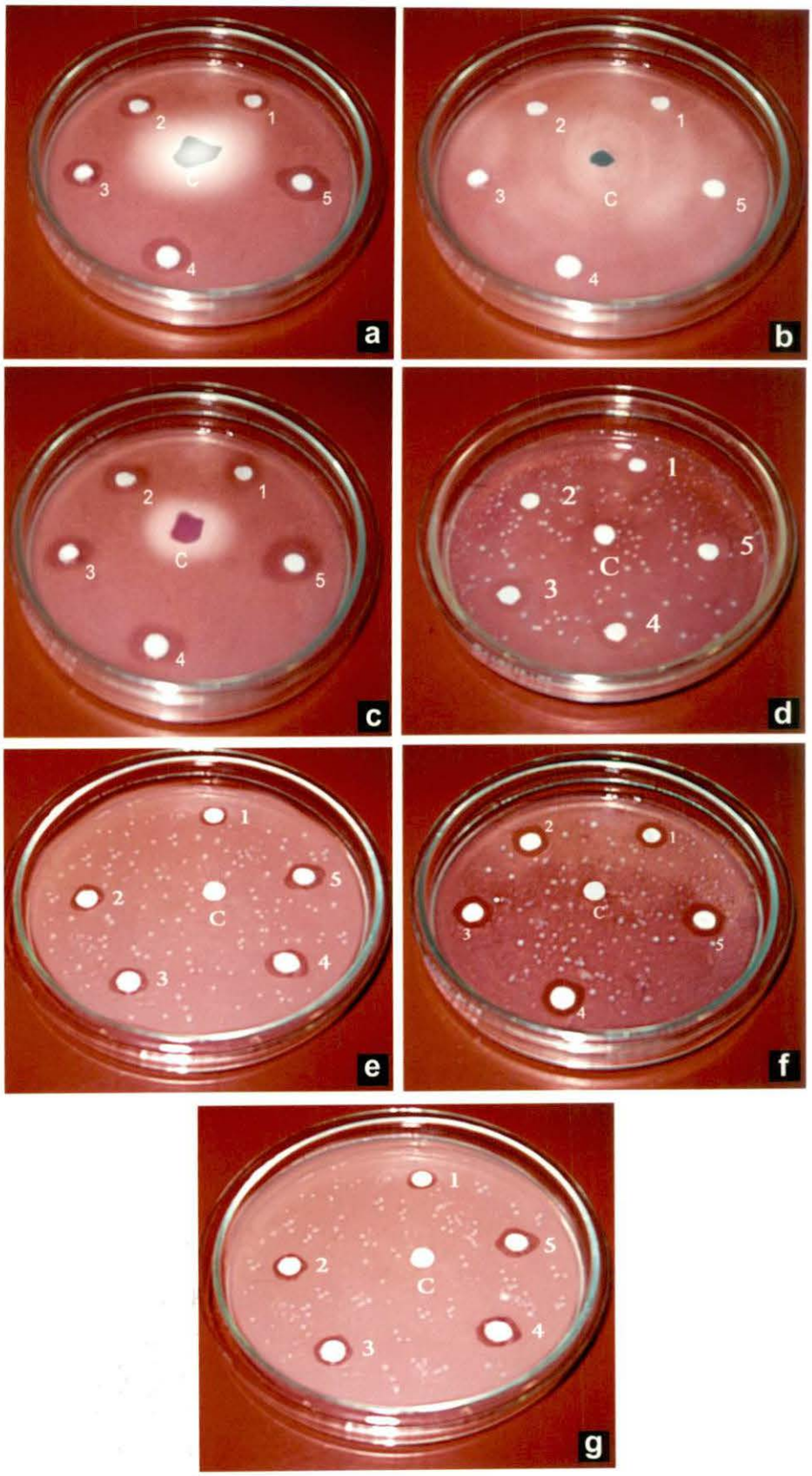


Plate 9: Disc diffusion test for anti microbial activity of compound I:(28 carbomethoxy lupane - [2,3-C]-**1′,2′,5′**-oxadiozole); at five different concentrations* against (a) *Colletotrichum gloeosporioides* (b) *Curvularia eragrostidis* (c) *Fusarium equiseti* (d) *Ralstonia solanacearum* (e) *Pseudomonas syringae* (f) *Erwinia carotovora*. (g) *Xanthomonas* sp.

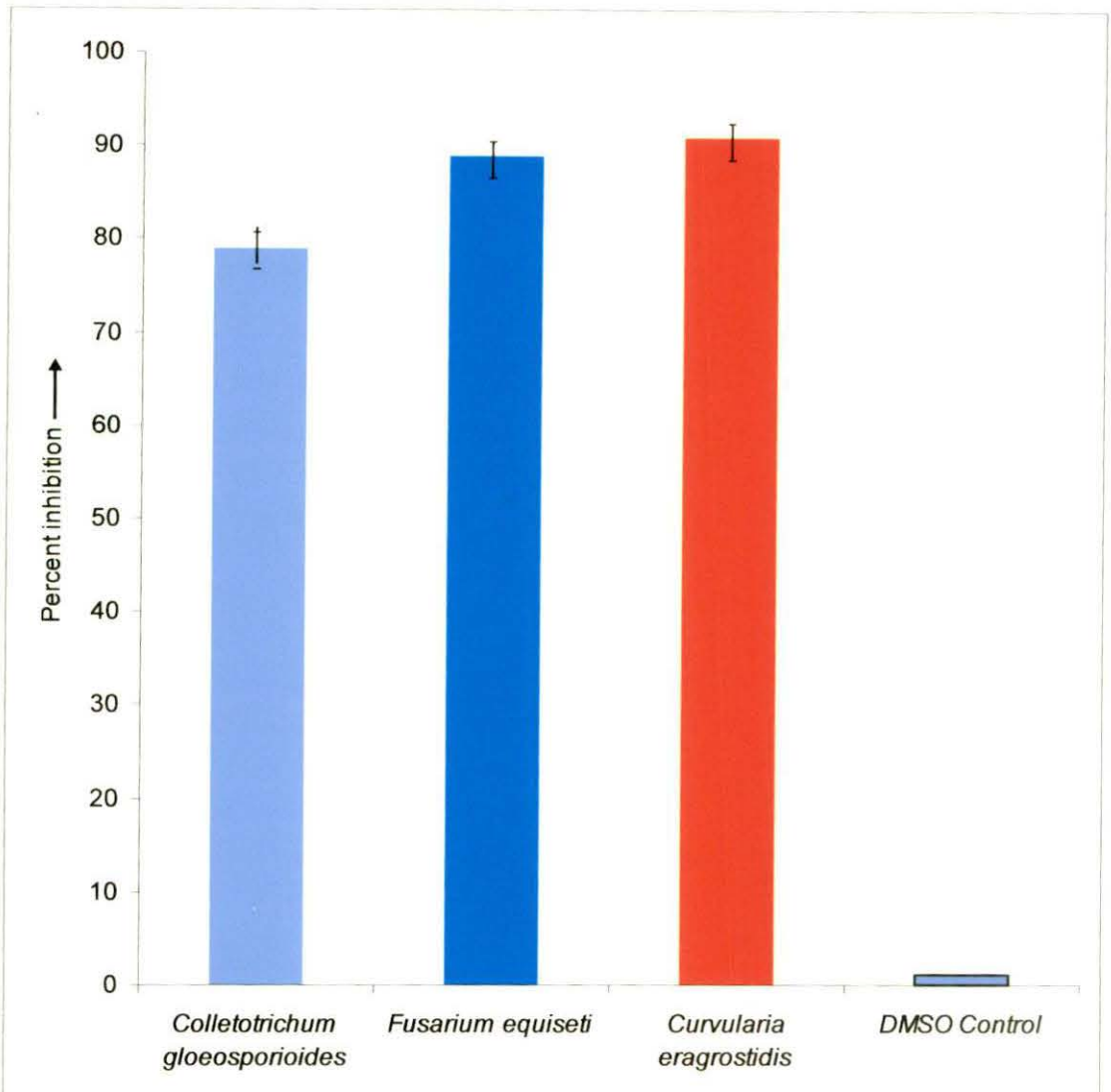


Fig. 36: Percent inhibition of spore germination at 300 ppm concentration of compound I (28-carbomethoxy lupane[2,3-C]-1',2',5'-oxadiazole).

From the results presented in table-3.8 it was evident that compound-G (2α-Bromo methyldihydrobetulonate) could inhibit more than 90% of spore germination at higher concentrations (400ppm & 500ppm) tested. More than 70% inhibition was also recorded at the lowest concentration (100ppm) tested. In disc diffusion test Compound-G could check *Colletotrichum gloeosporioides* and *Fusarium equiseti* but *Curvularia eragrostidis* was not controlled even at highest concentration (500ppm) tested. Compound-G controlled the growth of all the four bacteria (*Ralstonia solanacearum, Xanthomonas sp, Pseudomonas syringae* and *Erwinia carotovora*) at the five concentrations tested. But significant inhibition zone (more than 1.0 cm in diameter) was found at 300ppm concentration and above. In case of *Erwinia carotovora* and *Xanthomonas sp* more than 1.0 cm diameter of inhibition zones were observed at 200ppm concentration. But *Ralstonia solanacearum* and *Pseudomonas syringae* could be controlled significantly at all the five concentrations tested. Diameter of inhibition zones were more than1.0cm.

Compound H (28-carbomethoxylup-1(2)-en-3-one) reduced germination of spores of all the three fungi (*Colletotrichum gloeosporioides*, *Fusarium equiseti* and *Curvularia eragrostidis*) even at lowest concentration(100ppm). But more than 95% germination was checked at 500ppm concentration. In disc diffusion test growth of *Colletotrichum gloeosporioides* and *Fusarium equiseti* was controlled by the compound-H but the growth of *Curvularia eragrostidis* could not be controlled even at 500ppm concentration. The compound showed selective antifungal activity.

Compound-H showed best antibacterial activity against *Pseudomonas syringae.* At 100ppm concentration of the compound diameter of inhibition zone was 1.0cm. The other three bacteria showed moderate antibacterial activity. *Xanthomonas sp* and *R. solanacearum* showed low diameter of inhibition zone (0.5cm and 0.7cm respectively).

Unlike the other three compounds (F,G,H), compound-I (28 Carbomethoxy lupan[2,3-C]-1́,2́,5́-oxadiozole) was less effective in controlling germination of spores. *But Curvularia eragrostidis* was controlled by the compound significantly. About 85% of spores did not germinate at 100 ppm concentration of the compound-I. Whereas nearly 16% spores of *Colletotrichum gloeosporioides* and *Fusarium equiseti* could not

germinate at that concentration. Thus compound-I showed more effectiveness towards the inhibition of spore germination of *Curvularia eragrostidis* than *Colletotrichum gloeosporioides, Fusarium equiseti.* In contrast to the germination of spores, when growth of *Curvularia eragrostidis* was tested against five different concentrations of compound-I, no inhibition zone was found. This indicated that although spore germination of *Curvularia eragrostidis* could be controlled but mycelia growth was not controlled by the compound. The other two fungi showed significant zone of inhibition in disc diffusion test and thus showed the effectiveness of the compound to control the growth of two fungi mentioned. Compound-I showed antibacterial activity at higher concentrations (300ppm, 400ppm and 500ppm) towards all the four bacteria (*Ralstonia solanacearum, Xanthomonas sp, Pseudomonas syringae,and Erwinia carotovora*). Thus the compound I showed moderate antibacterial activity.

On the basis of phytotoxicity test 100ppm concentration of compound F,G,H I were not phytotoxic. Hence the compounds which are effective in controlling patl at 100ppm concentration and also not phytotoxic may be recommended for conti the pathogens *in vivo* or in field condition.

Section 3: References

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