CHAPTER IV

EXPERIMENTAL & REFERENCES

Melting points are uncorrected. Petroleum ether used during the investigation had the b.p. 60-80^{0}C range. IR spectra were taken in nujol or KBr on Beckman IR–20 or Shimadzu FT-IR 8300 spectrophotometer. UV spectra were determined in methanol on Shimadz–UV 240. ^{1}H NMR spectra were recorded on Bruker WH-400, Varian XL-200 or 300 MHz Bruker avance FT-NMR spectrometers using $CDCl_3$ solution containing TMS as reference. ^{13}C NMR spectra were recorded on Bruker WH-270 or 300 MHz Bruker avance FT-NMR spectrometer using $CDCl_3$ solution containing TMS as reference. Mass spectra were recorded on Varian Mat 711(70 ev), JMS-D 300(70 ev) or JEOL-AccuToF JMS-T100LC by EI/CI method. All the rotations were taken in $CHCl_3$ solution. Column chromatography was performed over silica gel (60–120 mesh, BDH). TLC was performed on chromatoplate of silica gel G (Glaxo and BDH) and the spots were located by exposing to iodine vapour.

Jones' oxidation of cholesterol : preparation of cholest-4(5)-en-2,6-dione (26):

To a solution of cholesterol (1 g) in pure acetone (150 mL) Jones' reagent was added dropwise with shaking until a faint orange colour persisted. The mixture was kept for 1hour, diluted with water and extracted with ether. The ether layer was washed with water, dried (Na_2SO_4) and then ether was evaporated out. The residue (0.9 g) dissolved in minimum volume of toluene was chromatographed over silica gel (20 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 5).

Table 5

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Petroleum ether + ethyl acetate (80:20)	4-10	White solid (≈ 0.8 g)	117-19°C

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

Examination of fractions 4-10 (Table 5) : Isolation of cholest-4(5)-en-2,6-dione (26)

Fraction 4-10 (Table 4) of same R_f value were combined and was purified by repeated crystallization from chloroform-methanol mixture to afford a white crystal, m.p. 121-22^{0}C, identified as cholest-4(5)-en-2,6-dione 26 by a comparison with authentic specimen (Lit. [16] m.p. 123^{0}C, UV- 252 nm [146-147]).

Analysis report	%C	%H
Found	81.19	10.38
Calculated for $C_{27}H_{42}O_2$	81.41	10.55

IR: ν_{max}^{Nujol} 1690 cm^{-1} (α, β-unsaturated ketone)

UV: λ_{max}^{MeOH} 250 nm (ϵ = 11000)

Treatment of cholest-4(5)-en-2,6-dione (26) with lithium-ethylenediamine: Isolation of cholestane (27) and cholestane-3β-ol (28)

Small pieces of lithium metal (0.8 g) were added at intervals to a solution of cholest-4(5)-en-2,6-dione 26 (0.8 g) dissolved in dry ethylenediamine (200 mL) and the mixture refluxed for 2 hours under nitrogen blanket. The reaction mixture was cooled and treated with solid NH_4Cl to destroy excess of lithium. It was acidified with dil HCl and extracted with ether. The ether solution was washed with water till neutral and dried over anhydrous Na_2SO_4. Evaporation of the solvent (ether) furnished a residue. The residue (0.8 g) was dissolved in minimum volume of toluene and chromatographed over silica gel (25 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 6).

Table 6

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-5	Solid	--
Pet. ether + ethyl acetate (80:20)	6-9	Nil	--
Pet. ether + ethyl acetate (60:40)	10-12	Nil	--
Pet. ether + ethyl acetate (40:60)	13-15	Nil	--
Pet. ether + ethyl acetate (20:80)	16-20	Solid	78-80^{0}C

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

Examination of fractions 1-5 (Table 6) : Isolation of cholestane (27)

Fractions 1-5 (Table 6) showed homogeneity on a TLC plate, hence these were combined (≈ 0.3 g) and after repeated crystallizations from chloroform-methanol mixture it afforded a white crystal, m.p 81-82^{0}C, m.f. $C_{27}H_{48}$, $[\alpha]_D$ + 27.9^0 (Lit. [94] m.p. 80-83^0, $[\alpha]_D$+ 28.7^0] was identified as cholestane 27.

Analysis report	%C	%H
Found	87.02	12.87
Calculated for $C_{27}H_{48}$	87.10	12.90

IR : No characteristic absorption. Fig. 1

Examination of fractions 16-20 (Table 6) : Isolation of cholestane-3β-ol (28)

The polar fractions 16-20 (Table 6) of same R_f value in TLC were combined (0.35 g) and crystallized from chloroform-methanol mixture when fine crystals, m.p. 140-141^{0}C, $[\alpha]_D$+24^0, IR: 3400 cm^{-1}, m.f. $C_{27}H_{47}OH$, was identified as cholestane-3β-ol 30 by comparison (mixed m.p, Co -IR, Co-TLC) with an authentic specimen of cholestane-3β-ol 28 [95] (Lit. [148] m.p. 142^0, $[\alpha]_D$ +24^0].

Analysis report	%C	%H
Found	83.10	12.50
Calculated for $C_{27}H_{48}O$	83.40	12.50

IR: ν_{max}^{Nujol} 3400 cm^{-1} Fig. 2

TNM test : No yellow colouration.

Acetylation of (30) : Preparation of cholestan-3β-yl acetate (29):

Compound (0.1g) was treated with acetic anhydride (2 ml) and pyridine (2 ml) and the mixture was heated on a water bath for four hours. The reaction mixture was cooled and then poured into ice cold water and worked up in the usual manner. The solid obtained after usual work up was crystallized from a mixture of chloroform-methanol to afford crystals of acetate m.p. 110-11^{0}C, $[\alpha]_D$ +10^o which was found to be identical (mixed m.p., Co-IR and Co-TLC) with an authentic specimen of cholestan-3β-yl acetate (Lit. [148], m.p. 111^{0}C $[\alpha]_D$ +13^0).

Analysis report	%C	%H
Found	80.78	11.50
Calculated for $C_{29}H_{50}O_2$	80.93	11.62

IR: ν_{max}^{Nujol} 1730, 1250 cm^{-1} (-OCOCH$_3$).

Treatment of hexane-2,5-dione (32) with lithium-ethylenediamine:

The product obtained after usual work up was separated by column chromatography and purified by double distillation. The less polar fractions eluted with petroleum ether (0.2 g) showed b.p 68^{0}C, m.f. C_6H_{14} (Lit. [96] b.p. 69^{0}C) was identified as n-hexane 31.

Analysis report	%C	%H
Found	83.70	16.30
Calculated for C_6H_{14}	83.72	16.30

The polar fraction eluted with petroleum ether : ethyl acetate (1:4) (0.15g) showed b.p. 218^{0}C, $[\alpha]_D$ -17.6^0, IR: 3400 cm^{-1} m.f. $C_6H_{14}O_2$, was identified as hexane-2,5-diol 30.

Analysis report	%C	%H
Found	72.78	11.90
Calculated for $C_6H_{14}O_2$	72.88	11.86

Treatment of sodium-ethylenediamine on dimedone (5,5-dimethyl cyclohexane-1,3-dione; 33): Isolation of 34

Dimedone (1 g) was dissolved in dry ethylenediamine (250 mL). To the solution small pieces of sodium metal (1 g) were added at intervals and the mixture was refluxed under nitrogen atmosphere for two hours as usual. The residue (0.7 g) after usual work up was dissolved in minimum volume of toluene and chromatographed over silica gel (35 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 7).

Table 7

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Pet. ether + ethyl acetate (80:20)	4-6	Nil	--
Pet. ether + ethyl acetate (60:40)	7-9	Nil	--
Pet. ether + ethyl acetate (40:60)	11-12	Oil	--
Pet. ether + ethyl acetate (20:80)	13-15	Oil	--
Ethyl acetate	16-20	White solid	176-77^{0}C

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

Examination of fractions 16-20 (Table 7) : Isolation of (34)

Fraction 16-20 (Table 7) showed homogeneity on a TLC plate, hence these were combined (≈ 0.3 g) which after repeated crystallization from chloroform-methanol mixture to afford a white crystal, m.p. 180-82^{0}C which was identified as 34.

Analysis report	%C	%H
Found	77.97	08.92
Calculated for $C_{16}H_{22}O_2$	78.05	08.94

IR: ν_{max}^{Nujol} 3160, 1550, 1650 (weak intensity), 1480, 1060, 1140, 860 cm^{-1}. Fig. 3

UV: λ_{max}^{MeOH} 275 nm (€ = 7500) Fig. 4

^{1}H NMR ($CDCl_3$): 1.09 (s, 12H, 4t-CH_3), 2.32 (s, 4H, 2-CH_2), 2.41 (d, 4H, 2 x CH_2, J = 1 Hz), 6.31 (s, 2H, 2 x CH) ppm. Fig. 5

Mass : m/z at 246 $[M]^+$, 231, 218, 203, 190, 134, 91, 83 (base peak). Fig. 7

^{13}C NMR: Fig. 6

Treatment of sodium-ethylene diamine on benzil (41) : Isolation of 2,3-diphenyl pyrazine (42)

Small pieces of sodium metal (0.58 g) were added at intervals to a solution of benzil 41 (0.58 g) in dry ethylene diamine (150 mL) and refluxed for two hours under nitrogen blanket. The reaction mixture was cooled and treated with solid NH_4Cl to destroy excess of sodium. It was acidified with dilute hydrochloric acid with water till neutral and dried over anhydrous Na_2SO_4. Evaporation of solvent (ether) furnished a gummy residue (0.2 g). The residue was dissolved in minimum volume of toluene and chromatographed over silica gel (15 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 8).

Table 8

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Pet. ether + ethyl acetate (80:20)	4-7	Oil	--
Pet. Ether + ethyl acetate (60:40)	8-11	Solid ($\approx$ 0.15 g)	116-17^{0}C

Further elution with more polar solvents did not afford any solid material.

Examination of fraction 8-11 (Table 8): Isolation of 2, 3-diphenyl pyrazine (42)

The fractions 8-11 (Table 8) showed homogeneity on a TLC plate, hence these were combined (≈ 0.15 g) which after crystallization from chloroform-methanol mixture afforded pale yellow crystals of compound 42, m.p. 119^{0}C, which was identified as 2,3-diphenyl pyrazine 42 (Lit. [134] m.p. 118-19^{0}C).

Analysis report	%C	%H
Found	82.71	05.11
Calculated for $C_{16}H_{12}N_2$	82.76	05.17

IR: ν_{max}^{Nujol} 1600, 1100 cm^{-1} (aromatic ring). Fig. 8

UV: λ_{max}^{MeOH} 263 nm (€ = 5712)

385 nm (€ = 1009)

TNM test: Negative

^{1}H NMR ($CDCl_3$): 7.25-7.33 (6s, 6H) Fig. 10

7.43-7.65 (4s, 4H)

8.61 (s, 2H) ppm

^{13}C NMR: Fig. 11

Mass: m/z at 232 $[M]^+$, 231 (base peak),

205, 204, 176, 103, 76. Fig. 9

Treatment of sodium-ethylene diamine on 4,4′-dibromobenzil (43): Isolation of pyrazine derivative of 4,4′-dibromobenzil (44)

Small pieces of sodium metal (0.58 g) were added at intervals to a solution of 4,4′-dibromo benzil 43 (0.58 g) in the dry ethylene diamine (150 mL) and refluxed for two hours under nitrogen blanket. The reaction mixture was cooled and treated with solid NH_4Cl destroy excess of sodium. It was acidified with dilute hydrochloric acid with water till neutral and dried over anhydrous Na_2SO_4. Evaporation of solvent (ether) furnished a gummy residue (0.2 g). The residue was dissolved in minimum volume of toluene and chromatographed over silica gel (15 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 9).

Table 9

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Pet. ether + ethyl acetate (80:20)	4-7	Oil	--
Pet. Ether + ethyl acetate (60:40)	8-11	Solid (≈ 0.15 g)	119-20^{0}C

Further elution with more polar solvents did not afford any solid material.

Examination of fractions 8-11 (Table 9): Isolation of pyrazine derivative of 4,4'-dibromobenzil (44)

The fractions 8-11 (Table 9) showed homogeneity on a TLC plate, hence these were combined (≈ 0.15 g) which after crystallization from chloroform-methanol mixture afforded pale yellow crystals of compound 44, m.p. 119^{0}C, which was identified as pyrazine derivative of 4,4'-dibromo benzil 44 (Lit. [134] m.p. 118-19^{0}C).

Analysis report	%C	%H
Found	39.10	02.21
Calculated for $C_{16}H_{10}N_2Br_2$	39.06	02.07

IR: ν_{max}^{Nujol} 1600, 1100 cm^{-1} (aromatic ring) Fig. 12

UV: λ_{max}^{MeOH} 263 nm (€ = 5712)

385 nm (€ = 1009)

TNM test: Negative

^{1}H NMR ($CDCl_3$): 7.23-7.50 (8s, 8H) Fig. 13

8.51 (s, 2H) ppm

^{13}C NMR: Fig. 14

Isolation of lupanone from *Xanthoxylum budrunga*

Five kilograms of air dried finely powered bark of *Xanthoxylum budrunga* [2] was soxhlated for 48 hours with toluene. The extract was cooled and then solvent was distilled off. The residue left was dissolved in minimum volume of toluene chromatographed over deactivated alumina column and the petroleum ether eluent collected. Petroleum ether was distilled off and a solid residue (12 g) was obtained. The solid on rechromatography over deactivated alumina afforded lupenone (1 g) and lupeol on petroleum ether: toluene (4:1) elution. Petroleum ether fractions were purified by repeated crystallizations from chloroform-methanol mixture. 0.8 g of lupenone, m.p. 170-72^{0}C, $[\alpha]_D$ +56.1^0, was obtained and found to be identical with authentic sample of lupanone (Co-TLC, mixed m.p and Co-IR) (Lit. [149] m.p. 170.5-72^{0}C, $[\alpha]_D$ +57.6^0].

IR: ν_{max}^{Nujol} 1705 (>C=O), 1630, 880 cm^{-1} (>C=CH$_2$).

Autoxidation of lupanone (42) : Isolation of diosphenol

Lupanone (0.6 g) isolated from bark of *Xanthoxylum budrunga* suspended in potassium tertiary butoxide (prepared from 6 g of potassium and 60 mL of tertiary butanol) was shaken in a stream of oxygen for two hours. The reaction mixture was then diluted with water and then 6*N* hydrochloric acid was added till the solution was acidic. It was then extracted with chloroform (100 ml) and the combined extract was dried (Na_2SO_4) and the solvent was removed under reduced pressure to yield a colorless solid which after crystallization from chloroform-methanol mixture afforded colorless crystals (0.5 g) of m.p. 188-89^{0}C, λ_{max} 272 nm, $[\alpha]_D$ +70.3^0, (Lit. [150] m.p. 187-90^{0}C, $[\alpha]_D$ +70.9^0]. It gave positive ferric chloride test and TNM test.

IR: ν_{max}^{Nujol} 3640 (-OH), 1670, 1650, 860 cm^{-1}.

Treatment of sodium-ethylenediamine on 2,3-diketolupane (45): Isolation of pyrazine derivative (48) of lupane (47)

Diosphenol (0.4 g) was dissolved in dry ethylene diamine (120 mL). To the solution small pieces of sodium metal (0.4 g) were added at intervals and the mixture was refluxed under nitrogen atmosphere for two hours as usual. The residue (≈ 0.37 g) after usual work up was dissolved in minimum volume of toluene and chromatographed over silica gel (20 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 10).

Table 10

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Pet. ether + ethyl acetate (80:20)	4-6	Nil	--
Pet. Ether + ethyl acetate (60:40)	7-10	White Solid	210-11^{0}C

Further elution with more polar solvents did not afford any solid material.

Examination of fractions 7-10 (Table 10): Isolation of (48)

The fractions 7-10 (Table 10) of same R_f value were combined (≈ 0.35 g) and crystallized from chloroform-methanol mixture to afford fine crystal, identified as pyrazine derivative 48 of lupene 47, m.p. 213-14^{0}C, $[\alpha]_D$ +24^0.

Analysis report	%C	%H
Found	83.40	10.31
Calculated for $C_{32}H_{48}N_2$	83.48	10.43

IR: ν_{max}^{Nujol} 1650, 1430, 1120 cm^{-1} (heterocyclic ring) and 1630, 990, 825 cm^{-1} Fig. 15

UV: λ_{max}^{MeOH} 272 nm (€ = 5700)

278 nm (€ = 5600)

TNM test was positive

Mass: m/z 460 $[M]^+$, 445 $[M\text{-}CH_3]^+$ (base peak), 438, 377, 350, 257, 256, 241, 229, 191, 189, 175, 149, 121, 109, 55. Fig. 16

^1H NMR ($CDCl_3$): 0.81 (s, 6H, 2t-CH_3), 1.00, 1.11, 1.28, 1.30 (4s, 16H, 4t-CH_3), 1.27 (CH-CH_3), 2.45 and 3.04 (2d, 2H, -CH_2, J= 16 Hz), 4.6 (m, 2H, =CH_2), 4.71 (d, 2H, 29-CH_2, J=3 Hz), 8.27 and 8.405 (2d, 2H, J = Hz 2 pyrazine ring protons) ppm. Fig. 17

^{13}C NMR: Fig. 18

Isolation of betulinic acid

Dried and powdered trunk bark of *Bischofia javanica* (2 kg) was extracted with toluene in a soxhlet apparatus for twenty hours. Benzene was distilled off and the gummy residue (9 g) was taken up in ether (1 lit). 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 acidified (1 lit) when some insoluble solid separated out. The acidified portions was extracted with ether, washed with water till neutral and then dried (Na_2SO_4). Ether was removed when a gummy residue (2 g) of betulinic acid was obtained.

Esterification of betulinic acid: Preparation of methyl betulinate

To the crude acid (3 g) dissolved in ether (500 ml) was added a solution of diazomethane in ether prepared from nitrosomethyl urea (1.5 g) and was kept overnight. Next day excess of diazomethane was destroyed with acetic acid (1 ml). The ether solution was washed with water, 10% sodium bicarbonate solution and again with water till neutral and then dried (Na_2SO_4). Evaporation of the ether yielded a gummy residue (2 g). This crude ester (1 g) dissolved in toluene (12 mL) was placed over a column of alumina (100 g, deactivated with 4 ml of 10% aqueous acetic acid) developed with petroleum ether and was eluted with the following solvents (Table 11).

Table 11

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-4	Oil	--
Pet. ether +ethyl acetate (80:20)	5-8	Nil	--
Pet. ether + ethyl acetate (60:40)	9-14	White Solid	217-19^{0}C

Further elution with more polar solvents did not afford any solid material.

Examination of fractions 9-14 (Table 11): Isolation of methyl betulinate

Solid obtained from the fractions 9-14 (Table 11) were combined (1.7 g), and crystallized from a mixture of chloroform and methanol to afford colorless needles of methyl betulinate m.p. 223-24^{0}C, $[\alpha]_D$+5^0 (Lit. [151] m.p. 224-25^{0}C, $[\alpha]_D$ +5^0), identical with authentic sample (mixed m.p., Co-TLC and Co-IR).

Hydrogenation of methyl betulinate : Preparation of methyl dihydrobetulinate

Methyl betulinate (1.7 g), dissolved in a mixture of ethyl acetate and acetic acid (50 ml each) was shaken in an atmosphere of hydrogen in presence of PtO_2 catalyst (0.1 g) for three hours until absorption of hydrogen ceased. Ethyl acetate was removed by distillation and the solution was diluted with water whereby a white solid (1.8 g) separated out which was collected by filteration. Crystallisation from a mixture of chloroform and methanol furnished colorless needles of m.p. 236-38^{0}C, identical (mixed m.p., Co-IR and Co-TLC) with authentic specimen of dihydromethyl betulinate.

IR: ν_{max}^{Nujol} 3560 (-OH), 1730 cm^{-1} (-$COOCH_3$).

Jone's oxidation of methyl dihydrobetulinate : Preparation of methyl dihydrobetulinate

To a solution of methyl dihydrobetulinate (1.7 g) in pure acetone (250 mL) was added Jone's reagent dropwise with shaking until a faint orange color persisted. The mixture was kept at room temperature for 1 hour, diluted with water and extracted with ether. The ether layer was washed thoroughly with water, dried (Na_2SO_4) and the ether evaporated. The residue (1.6 g) dissolved in minimum volume of toluene was chromatographed over silica gel (40 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 12).

Table 12

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-4	Nil	--
Pet. ether + ethyl acetate (80:20)	5-15	White Solid (≈ 1.6 g)	196-99^{0}C

Further elution with more polar solvents did not afford any solid material.

Fractions 5-15 (Table 12) were combined and on crystallization from chloroform-methanol mixture furnished needle shaped crystals of compound, m.p. 202-4^{0}C, $[\alpha]_D$ +8^0, identical with an authentic samples of methyl dihydrobetulonate (mixed m.p., Co-IR and Co-TLC).

IR: ν_{max}^{Nujol} 1730 (-$COOCH_3$), 1708 cm^{-1}.

Autoxidation of methyl dihydrobetulonate : Preparation of diosphenol

Methyl dihydrobetulonate (1.5 g) suspended in potassium tersiary butoxide in tertiary butanol (prepared from 5 g potassium and 150 ml of tertiary butanol) was shaken in a stream of oxygen for 75 minutes. The residue (1.35 g) after usual work up was dissolved in minimum volume of benzene and chromatographed over silica gel (35 g) developed with petroleum ether and was eluted with the following solvents (Table 13).

Table 13

Eluent	Fractions 50 mL each	Residue on evaporation	m.p. after crystallization
Petroleum ether	1-4	Nil	--
Pet. ether + ethyl acetate (80:20)	5-15	Solid (≈ 1.2 g)	126-29^{0}C

Further elution with more polar solvents did not afford any solid material.

Fractions 5-15 (Table 13) of same R_f value were combined and on crystallization from a mixture of chloroform-methanol afforded colourless crystals, m.p. 131-32^{0}C, $[\alpha]_D$ -1.94^0 (Lit. [152] m.p. 131-33^{0}C, $[\alpha]_D$ -1.96^0). It gave positive ferric chloride coloration for diosphenol and was identified as 2,3 diketo dihydro betulanate.

UV: λ_{max}^{MeOH} 271 nm (ϵ = 7829)

IR: ν_{max}^{Nujol} 3460 (-OH), 1730 (-$COOCH_3$), 1670, 1650, 860 cm^{-1}.

Treatment of sodium-ethylene diamine on methyl 2,3-diketo dihydrobetulonate (49) : Isolation of 50

Diosphenol 49 (0.5 g) was dissolved in dry ethylene diamine (150 ml). To the solution small pieces of sodium metal (0.5 g) were added to it at intervals under nitrogen atmosphere. The reaction mixture was refluxed for two hours under nitrogen blanket. The residue (≈ 0.43 g) obtained after usual work up was dissolved in minimum

volume of toluene and chromatographed over silica gel (20 g). The chromatogram was developed in petroleum ether and eluted with the following solvents (Table 14).

Table 14

Eluent	Fractions 50 mL each	Residue On evaporation	m.p. after crystallization
Petroleum ether	1-3	Nil	--
Pet. ether + ethyl acetate (80:20)	4-6	Nil	--
Pet. Ether + ethyl acetate (60:40)	7-10	White Solid (≈ 0.4 g)	144-45^{0}C

Further elution with more polar solvents did not afford any solid material.

Fraction 7-10 (Table 14) of same R_f value were combined (≈ 0.4 g) and crystallized from chloroform-methanol mixture to afford fine crystals, identified as compound 50, m.p. 220^{0}C.

Analysis report	%C	%H
Found	78.25	09.73
Calculated for $C_{33}H_{50}O_2N_2$	78.26	09.88

IR: ν_{max}^{Nujol} 1710-20 (-COOMe), 1650-70, 1430, 1120 cm^{-1}. Fig. 19

UV: λ_{max}^{MeOH}	272 nm (€ = 5712) 278 nm (€ = 5603)	Fig. 20

TNM test: No yellow colouration.

Mass:	m/z 506 $[M]^+$, (base peak), 491, 463, 447, 432, 431, 258, 256, 241, 191, 187, 175, 159, 147, 133, 95, 55.	Fig. 21
^{1}H NMR ($CDCl_3$):	0.82-1.305 (5s, 15H, 5t-CH_3), 0.76 and 0.88 (2d, 6H, $CH(CH_3)_2$, J=7 Hz), 2.48 and 3.04 (2d, 2H, 1-CH_2, J= 16 Hz) 8.27 and 8.411 (2d, 2H, pyrazine ring proton, J=3 Hz) 3.66 (s, 3H, -$COOCH_3$) ppm.	Fig. 22

Experimental related to biocidal activity of the isolated compounds

DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37°C for 48 h. The bacterial growth was confirmed by a change of yellow to purple colour. Bacterial nutrient media was prepared by using agar, beef extract and bacto peptone in distilled water and the pH of the solution (6.8-7.0) was adjusted. Culture media for fungal strains were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber. The antifungal activities between these compounds and Bavistan and antibacterial activity with Streptomycin were compared.

Seeds of rice (*Oryza sativa*), wheat (*Triticum aestirium*) and pea (*Pisum sativum*) were collected from local market. The assay seeds were shorted for uniformity of size and all damaged seeds were discarded. Before the bioassay seeds were washed with tap water and the surface were sterilized using NaCl (10% v/v) for 10 min followed by several washes in sterile distilled water. For testing phytotoxicity dehydrated ethanol was used as control. Bioassays were carried out using petridishes (90 mm diameter) containing a sheet of whatman filter paper as support. Test solutions (5 ml) was added to the filter paper in the petridish and dried completely *in vacuo* at 40°C. Five seeds from each category were placed on the filter paper and incubated for 7 days at 25°C in the dark. The effects of the pure compounds were determined by measuring the elongation of roots and averaged for each concentration.

Collection of plants for extraction of botanicals

Plant parts (bark, root, stem, leaf etc.) of two different plants (*Biscofia javanica*, *Xanthoxylum budrunga*) were collected from foothills of Darjeeling and Terai region (West Bengal, India). The plants were selected on the basis of their folk medicinal value in these areas. The plants were collected, identified and voucher specimens have been deposited in the departmental herbarium of the Department of Botany, University of North Bengal.

Source of microbial cultures

Fungal and bacterial cultures used during the work were isolated from the field or procured from Microbial type culture collection (MTCC), Chandigarh, India. The details of the source of the fungal cultures are given in the Table 15.

Table 15. List of cultures used during the present study

Name of the microorganisms	Source	Number
Staphylococcus aureus	MTCC,Chandigarh, India	MTCC–96
Escherichia coli	MTCC,Chandigarh, India	MTCC–739
Pseudomonas aeruginosa	MTCC,Chandigarh, India	MTCC–2453
Escherichia coli	MTCC,Chandigarh, India	MTCC–2939
Colletrichum gloeosporioides	Plant pathology lab. NBU	Identification No. 5446.02
Fusarium equisiti	Plant pathology lab. NBU	Identification No. 6566.07
Curvularia eragrostidis	Plant pathology lab. NBU	Identified by Dr. A. Saha
Alterneria alternata	Plant pathology lab. NBU	Identification No. 7065.08
Colletotrichum camelliae	Plant pathology lab. NBU	Identified by Dr. A. Saha
Bacillus subtilis	Plant pathology lab. NBU	Identified by NBU
Enterobactor sp.	Plant pathology lab. NBU	Identified by NBU

Maintenance of stock cultures

Freshly prepared sterile slants of potato dextrose agar (PDA) medium were used for maintenanc and preservation of fungal cultures. After four to eight weeks of sub culturing, the fungal pathogens grown on sterile PDA slants were stored in two different conditions, viz. at room temperature (25°C) and in refrigerator (4-8°C). Apart from

weekly transfer for experimental purpose, the cultures were also examined at regular intervals under microscope.

Major Chemicals used

In addition to the common laboratory reagents, following chemicals were used during the work:

Chemicals	Company
$C_6H_{12}O_6$	Glaxo Laboratories (India) Ltd
$NaNO_3$	S d fine-CHEM limited
KCl	S d fine-CHEM limited
$MgSO_4$, $7H_2O$	Merck India Ltd.
$FeSO_4$, $7H_2O$	Merck India Ltd.
K_2HPO_4	Merck India Ltd.
$C_{12}H_{22}O_{11}$	Glaxo Laboratories (India) Ltd

Composition of media and solutions used

Some of the media/solutions used during the work are listed below along with their standard compositions.

Nutrient Agar (NA)

Components	Amount
Beef extract	1.0 g
Yeast	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15 g
Distilled water	1 lit

Potato Dextrose Agar (PDA)

Component	Amount
Potato	40 g
Dextrose	2 g
Agar agar	2 g
Distilled water	100 ml

Malt yeast Agar

Components	Amount
Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1.0 g
pH	7.0

Czapek concentrate

Components	Amount
$NaNO_3$	30.0 g
KCl	5.0 g
$MgSO_4$, 7 H_2O	5.0 g
$FeSO_4$, 7 H_2O	0.1 g
Distilled water	1.0 lit
K_2HPO_4	1.0 g
Yeast extracts	5.0 g
Sucrose	30.0 g
Agar	15.0 g

Staphylococcus aureus was grown in nutrient agar media at 37±1°C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Escherichia coli were grown in nutrient agar media at 37±1°C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Pseudomonas aeruginosa were grown in nutrient agar media at 37±1°C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Bacillus subtilis were grown in Czapek concentrate media at 25±1°C in an incubator for 7 days .The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 7 days old freshly grown cultures were used. Routine subcultures were made at 30 days intervals. *Colletotrichum gloeosporioides* were grown in nutrient agar media at 37±1°C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Fusarium equisiti were grown in nutrient agar media at 37±1°C in an incubator for 48 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Curvularia eragrostidis were grown in nutrient agar media at 37±1°C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals

Alterneria alternata were grown in nutrient agar media at 37±1°C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals

Colletotrichum camelliae was grown in nutrient agar media at 37±1°C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

Enterobactor sp. was grown in nutrient agar media at 37±1°C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term

maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

The biological activities of the compounds which extracted from *Biscofia javanica*, *Xanthoxylum budrunga* and Cork plants against fungal pathogens (*Colletrichum gloeosporioides*, *Fusarium equisitae*, *Curvularia eragrostidies*, *Alterneria alternate* and *Colletotrichum camelliae)*, bacteria (*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) and crops (rice, wheat, pea) were investigated. The experiments used in the study were done in the plant pathology laboratory Department of Botany, North Bengal University and Microbiology laboratory, Department of Microbiology, Raigang College (University College), Uttar Dinajpur.

Bacterial strains

Bacterial strains used in this study were purchased from Microbial type culture collections, Chandigarh, India. *Bacillus subtilis (MTCC No-2358), Escherichia coli (MTCC No-2939), Staphylococcus aureus (MTCC No-96) and Pseudomonas aeruginosa (MTCC No-2453)* were used for testing antibacterial activity. The species of bacteria were grown in nutrient agar media (pH-7.0) (HiMedia). The concentration of bacterial suspensions was adjusted to 10^8 cells/mL. The cultures were sub cultured routinely and used for experimental purposes.

Antibacterial assay

Antibacterial activity was measured using the method of diffusion disc plates on agar [94]. For nutrient agar 28 gm of media (HiMedia) was suspended in 1000 ml of distilled water according to the manufacturer's protocol. It was boiled to dissolve the medium completely at sterilized by autoclaving at 15 lbs pressure (121^0C for 15 min.). The nutrient agar contained peptic digest of animal tissue (5 gm), sodium chloride (5 gm), beef extract (1.5 gm), yeast extract (1.5 gm), agar (15 gm) and dissolved water (1000 ml). pH was adjusted to 7.0. The respective suspension (100 µL) with approximately 10^8 bacteria per millilitre was placed in petridishes over agar and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 µL of each sample (40 mg/mL).

Antibacterial sensitivity test by the disc diffusion method

One ml of 48 h old culture of the test bacterium was taken in a petridish of 90 mm diameter. Then 20 ml of sterile NA medium was poured in the petridish and shaken carefully to mix the bacterial suspension with the medium. The petridishes were allowed to cool. Filter paper discs of 5 mm diameter containing desired concentration of the test samples were placed on the surface of the solidified media and incubated at 37° C in an incubator for 48h. Diameter of the inhibition zones was noted. In control sets no chemicals were used in the filter paper discs but sterile distilled water was used to soak the filter papers. All tests were made in triplicates.

Fungal strains

The strains of fungi were obtained from microbiology department, North Bengal University. *Colletotrichum camelliae*, *Fusarium equisiti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* (identified by Dr. A. Saha, Plant Pathology Lab., NBU) were used for testing antifungal activity. The species of fungus were grown in potato dextrose media. The concentration of fungal suspensions was adjusted to 10^7 cells/mL. The cultures were sub cultured routinely and used for experimental purposes.

Antifungal assay

Antifungal activity was also measured using the method of diffusion disc plates on agar [95]. For preparation of PDA peeled potato was cut into small pieces and boiled in required volume of dissolved water. The mixture was filtered through muslin cloth and the extract was mixed with dextrose and agar. The resultant mixture was heated in order to dissolve. Finally the media was sterilized at 15 lbs (121^0C for 15 min.). Composition of the media was peeled potato (400 gm), dextrose (20 gm), agar (20 gm) and dissolved water (1000 ml). P^H was adjusted to 6.0. The fungal suspensions were adjusted to 10^8 as explained above.

Antifungal sensitivity test by the disc diffusion method

One ml of 48 h old culture of the test fungus was taken in a petridish of 90 mm diameter. Then 20 ml of sterile NA medium was poured in the petridish and shaken

carefully to mix the bacterial suspension with the medium. The petridishes were allowed to cool. Filter paper discs of 5 mm diameter containing desired concentration of the test samples were placed on the surface of the solidified media and incubated at 37^{0} C in an incubator for 48h. Diameter of the inhibition zones was noted. In control sets no chemicals were used in the filter paper discs but sterile distilled water was used to soak the filter papers. All tests were made in triplicates.

Phytotoxic assay

In order to show phytotoxic activities of the compounds solution of different concentrations of different compounds were prepared and applied to check germination of both root and shoot of the germinating seeds. Phytotoxicities of the isolated compounds were determined on the healthy seeds of wheat (Sonalika variety), rice (IR-20, Jaya variety) and pea purchased from Anup seed company, Bidhan Market, Siliguri, West Bengal. These healthy seeds were dipped in acetone-water suspensions of the compounds of different concentration(500 ppm, 250 ppm, 100 ppm) and incubated for 1,4 and 8 hours. The treated seeds of wheat, rice and pea were allowed to germinate on a mat of moist filter paper. The roots and shoots of germinated seeds were kept in a covered Petri plates. Each experiment was based on 80 seeds of each varieties or plants. After five days of incubation the germinated seeds (treated with compounds) were counted. Treated experimental sets were compared with that of control sets where no compounds were used to treat the seeds. Each experiment was repeated in triplicate. All apparatus and materials used were sterilized where necessary.

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