

Evaluation of Antibacterial Potentiality of a Cyclopenta Naphthalene Tetraol Terpenoid Isolated from *Curcuma caesia* Roxb.

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Research Article

Received: 29/11/2013

Revised : 14/12/2013

Accepted: 26/12/2013

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Keywords: *Curcuma caesia* Roxb., 2, 3, 4, 8a, 9, 9a - hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol, Antibacterial potentiality, Physicochemical characterization, Crop protectant.

ABSTRACT

The assessment of antibacterial potentiality of 2,3,4,8a,9,9a-hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a - decahydro - 1H - cyclopenta [b] naphthalene -1,2,3a,4a-tetraol was the primary objective of this paper followed by its physicochemical characterization. Chemical characterisation of 2, 3, 4, 8a, 9, 9a-hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol was done through UV, IR (FT-IR), HRMS and NMR spectroscopic techniques. Assessment of its antibacterial activity was performed using agar cup method and disc diffusion assay respectively. The antibacterial compound was terpenoid in nature and identified as 2, 3, 4, 8a, 9, 9a-hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol. The MIC values tested against different plant pathogenic fungi and bacteria are different. The MIC values of this compound was 365 µg/ml, 274 µg/ml, 389 µg/ml, 443 µg/ml tested against the bacterium *Serratia marcescens*, *Erwinia herbicola*, *Xanthomonas* sp. and *Arthrobacter chlorophenolicus* respectively. Best of our knowledge this was the first report of presence of 2, 3, 4, 8a, 9, 9a-hexamethyl-2, 3, 3a, 4, 4a, 5, 8, 8a, 9, 9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol in plants. Due to its antibacterial property it may function in plant defense or commercialize as an ecofriendly crop protectant.

INTRODUCTION

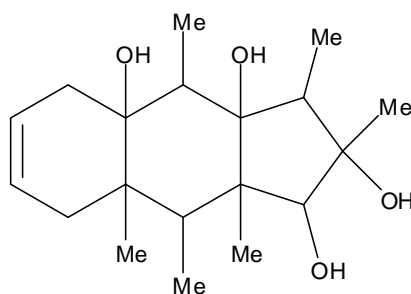
Curcuma caesia Roxb. (Black turmeric) of the family Zingiberaceae is a natural triploid, endemic and ethnomedicinally important plant. This plant was used by the tribal's of northeast India for its unique medicinal properties. There was a few report on the bioactive potentialities of black turmeric. So far eight metabolites have been isolated and characterised from *Curcuma caesia* Roxb. like Borneol, Borneol acetate, 1,8-Cineole, α-Curcumene, γ-Curcumene, β-Elemene, (E)-β-Ocimene, ar-Turmerone etc ^[1,2]. Here we report for the first time the presence of 2, 3, 4, 8a, 9, 9a - hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol (Figure 1) from the shade dried rhizome of *Curcuma caesia* Roxb. Best of our knowledge this compound has seem to be a novel one which was not reported earlier.

MATERIALS AND METHODS

Collection of plant material

Whole plant of *C. caesia* Roxb. was collected in the month of July 2010 from experimental garden of Department of Botany, University of Kalyani, and was identified in the Department of Botany, University of Kalyani, Nadia.

Figure 1: Structure of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.



Extraction and isolation of crude secondary metabolite content

2.5 kg shade dried rhizomes of black turmeric plant was powdered of approximately and extracted three times with 1 liter of 95% EtOH at room temperature to give an extract of 479 gms. The extract was evaporated under reduced pressure and a solid residual mass was obtained. The above obtained residual sample was subjected to repeated preparative thin layer chromatography using different solvent systems, e.g solvent system 1. Methanol (5%): benzene (95%) and solvent system 2. Chloroform (60%): benzene (30%): acetic acid (10%). Three homogeneous spots were collected in solvent system 2, having Rf values of 0.87, 0.79 and 0.75 respectively. The sample with Rf value 0.87 was taken up for further study. This sample was positive in Liebermann's Burchard test [4] and gave purple colour indicating its terpenoid nature. The compound had melting point of 91°C. The sample was then further analysed through various spectroscopic techniques like UV spectroscopy (UV- 1601PC, UV-Visible Spectrophotometer, Shimadzu), FT-IR spectroscopy (Perkin Elmer Spectrum- 1 Spectrophotometer), High Resolution Mass spectroscopy (JEOL- JMS 600 Instrument) and Nuclear Magnetic Resonance spectroscopy, ¹H & ¹³C (Bruker Avance- 400 Spectrometer) for its proper physicochemical characterization.

Antibacterial assay

Microorganisms, culture media and their incubating environment

The isolated sample were individually tested against a panel of microorganisms including Gram negative *Serratia marcescens* (MTCC NO. 7298) incubated at 30°C, *Erwinia herbicola* (MTCC NO. 3609) incubated at 37°C, *Xanthomonas* sp. (MTCC NO. 7444) incubated at 30°C and Gram positive *Arthrobacter chlorophenolicus* (MTCC NO. 3706) incubated at 28°C. All the bacterial strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The reference strains of bacteria were maintained on nutrient agar medium and LB medium slants at 4°C with a subculture period of 30 days.

Composition of the media

Details of composition of the media in which test microorganisms were grown are given in table 1.

Table 1: Composition of the media for test bacterium.

Constituents	Weight / Volume	Medium
Beef extract	1.0g	Nutrient agar medium (pH 7.0) After adjusting the pH, volume of the medium was adjusted to 1 liter by adding double distilled sterile water. Nutrient broth medium has the same composition without agar.
Yeast extract	2.0g	
Peptone	5.0g	
NaCl	5.0g	
Agar	15.0g	
Tryptone	10.0g	LB agar medium (pH 7.0) After adjusting the pH, volume of the medium was adjusted to 1 liter by adding double distilled sterile water. LB broth medium has the same composition without agar.
Yeast extract	5.0g	
NaCl	10.0g	
Agar	15.0g	

Preparation of McFarland standard

The turbidity standard was prepared by mixing 0.5 ml of 1.75% (w/v) BaCl₂.2H₂O with 99.5 mL of 1% H₂SO₄.BaSO₄ (v/v). The standard was taken in screw cap test tube to compare the turbidity. The bacterial culture of

selected strains were grown for 48- 72 hours and subsequently mixed with physiological saline. Turbidity was corrected by adding sterile saline until McFarland 0.5 BaSO₄ turbidity standard 10⁸ Colony Forming Unit (CFU) per ml was achieved. These inocula were used for seeding of the nutrient agar medium and LB medium respectively.

Disc diffusion assay

1 mg of the isolated sample was separately dissolved in 1 ml of propylene glycol and then the volume was adjusted to 10 ml by adding sterile water. The ultimate concentration reaches to 10³ µg/ ml and sterilized by filtration (0.22 µm millipore filter). The concentrations at 500 to 100 µg/ ml were taken in each case. The sterile paper discs (6 mm diameter) were saturated with 10 µl of the solution of the compound at a concentration of 500 to 100 µg/ml and placed on the inoculated agar of 10⁸ cfu/ml. Antibacterial tests were then carried out by disc diffusion method [5] using 100 µl of suspension containing 10⁸ CFU/ml of bacteria on nutrient agar medium and LB medium respectively. Negative controls were prepared using propylene glycol. Gentamicin (10 µg/ disc) was used as positive reference standards to determine the sensitivity of each bacterial species tested. The inoculated plates were incubated at 30^o C, 37^o C, 30^o C and 28^o C respectively for 48 h, 24 h, 48 h and 72 h. Antibacterial activity was evaluated by measuring the zone of inhibition and the diameters of these zones were measured in millimeters against the test organisms [6].

Determination of minimum inhibitory concentration

The minimal inhibitory concentration (MIC) values were studied for the bacteria strains, being sensitive to the compound in disc diffusion assay. The inocula of the bacterial strains were prepared from 24-72 hr broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The compound was dissolved in 1 ml of propylene glycol and then serial dilutions were made in order to obtain a concentration range from 500 to 100 µg/ml in 10 ml sterile test tubes containing nutrient broth and LB broth medium respectively. MIC values of the compound against bacterial strains were determined based on a micro well dilution method as previously described [7-8]. The plate was covered with a sterile plate sealer and then incubated at appropriate temperatures for 24 - 72 h at 30^o C, 37^o C, 30^o C and 28^oC respectively. Bacterial growth was determined by absorbance at 600 nm and confirmed by plating 10 µl samples, forming clear wells on nutrient agar medium or LB medium respectively. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. Each test in this study was repeated, at least, thrice.

RESULTS AND DISCUSSION

Chemical characterization of the isolated sample

The compound was reddish brown amorphous solid and was readily soluble in methanol. The melting point of the sample was 91^oC and it turned purple in Liebermann's Burchard test [3].

Bayer's test for presence of double or triple bond

In ~2-3 mg of the isolated compound in methanol was added in the very diluted alkaline solution of potassium permanganate. The purplish pink colour of the reaction mixture turns to brown indicating presence of an active unsaturation (double or triple bond) in the compound.

UV spectroscopy of the isolated sample

The methanolic spectrum of the sample showed λ_{max} at 364.5 nm, 337.5 nm, 333.00 nm, 214.00 nm and absorbance at = 0.0025, 0.0025, 0.0035, 2.2272 respectively (Figure 2).

IR (FT-IR) spectroscopy of the isolated sample

The IR spectrum of the sample showed ν- (cm⁻¹): 3339, 2966, 2924, 2879, 2858, 1669, 1448, 1377, 1057 and 1003 (Figure 3).

High Resolution Mass spectroscopy of the isolated sample

The mass of the sample was noted as to be (TOF MS ES⁺) 347.0627 (M+Na) (Figure 4).

Nuclear Magnetic Resonance spectroscopy of the isolated sample

¹H NMR (400 MHz, CDCl₃):

δ . 5.40 (1H, dt, $J = 6.8, 0.8$ Hz, Oliphinic H), 5.10 (1H, dt, $J = 6.8, 0.2$ Hz, Oliphinic H), 4.12 (2H, d, $J = 6.8$ Hz), 3.64 (1H, m), 2.6 (2H, br s), 2.10 (2H, m, HC=CH-CH₂), 2.0 (2H, m, CH=CH-CH₂), 1.68 (6H, s, CH₃), 1.66 (6H, s, CH₃), 1.60 (6H, s, CH₃), 1.36 (1H, m), 1.17 (1H, m) and 0.90 (1H, $J = 6.8$ Hz). (Figure 5).

¹³C NMR and DEPT- 135 (100 MHz, CDCl₃):

δ . 139.3, 131.6, 131.2, 124.7, 123.9, 123.4, 60.9, 59.1, 39.78, 39.5, 37.2, 29.2, 26.4, 25.7, 25.6, 25.4, 19.4, 17.6 and 16.2 (Figure 6 & 7).

Assessment of antibacterial potentiality

Antibacterial assay was performed with 2, 3, 4, 8a, 9, 9a -hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol against four plant pathogenic bacterium and the MIC value was 365 μ g/ml, 274 μ g/ml, 389 μ g/ml and 443 μ g/ml for the bacterium *Serratia marcescens* (MTCC NO. 7298), *Erwinia herbicola* (MTCC NO. 3609), *Xanthomonas* sp. (MTCC NO. 7444) and *Arthrobacter chlorophenolicus* (MTCC NO. 3706) respectively (Table 2).

Table 2: Antibacterial potentialities of 2, 3, 4, 8a, 9, 9a -hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.

Serial number	Bacterial strains	Concentration of the compound (MIC values)	Diameter of inhibition zone in mm
1	<i>Serratia marcescens</i>	365 μ g/ml	6.3
2	<i>Erwinia herbicola</i>	274 μ g/ml	5.2
3	<i>Xanthomonas</i> sp.	389 μ g/ml	4.7
4	<i>Arthrobacter chlorophenolicus</i>	443 μ g/ml	2.1

Interpretation of the structure of the isolated compound

The U.V spectrum of the compound in methanol showed the intense absorption peak (λ_{max}) at 217 nm, indicating the presence of unconjugated but alkyl substituted chromophoric double bond attached with auxochromic functionality (Figure-2). When I.R spectrum of this compound was recorded, characteristic strong absorption peaks in functional group region were also found. The broad and very strong absorption at 3339 cm^{-1} was assigned for aliphatic O-H stretching. Aliphatic C-H stretching was found at 2966 cm^{-1} (asymmetric) and 2824 cm^{-1} (symmetric). In addition, an absorption peak of moderate intensity at 1669 cm^{-1} was also observed indicating the presence of aliphatic C=C stretching [4] (Figure-3).

Figure 2: UV spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.

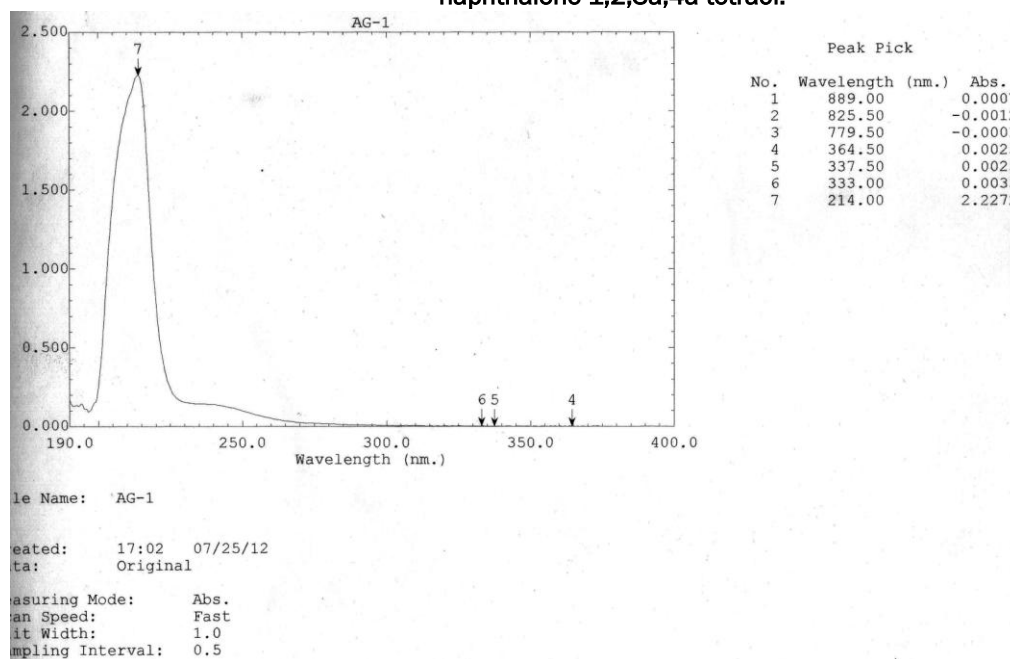


Figure 3: IR (FT-IR) spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.

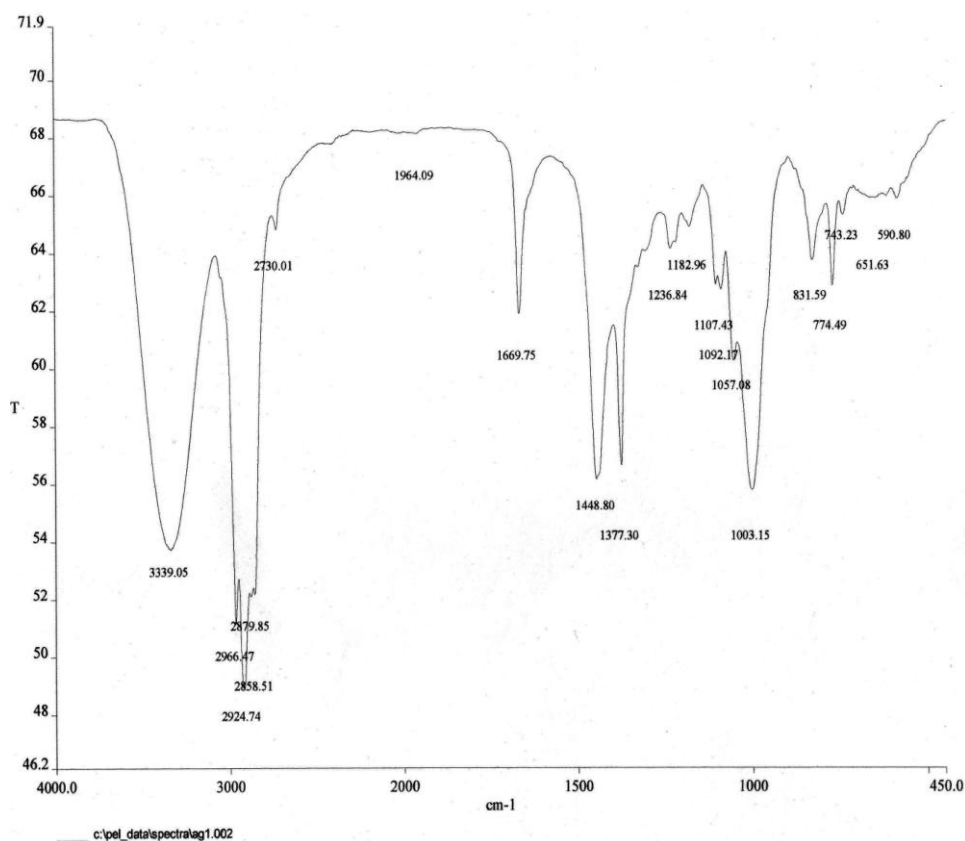
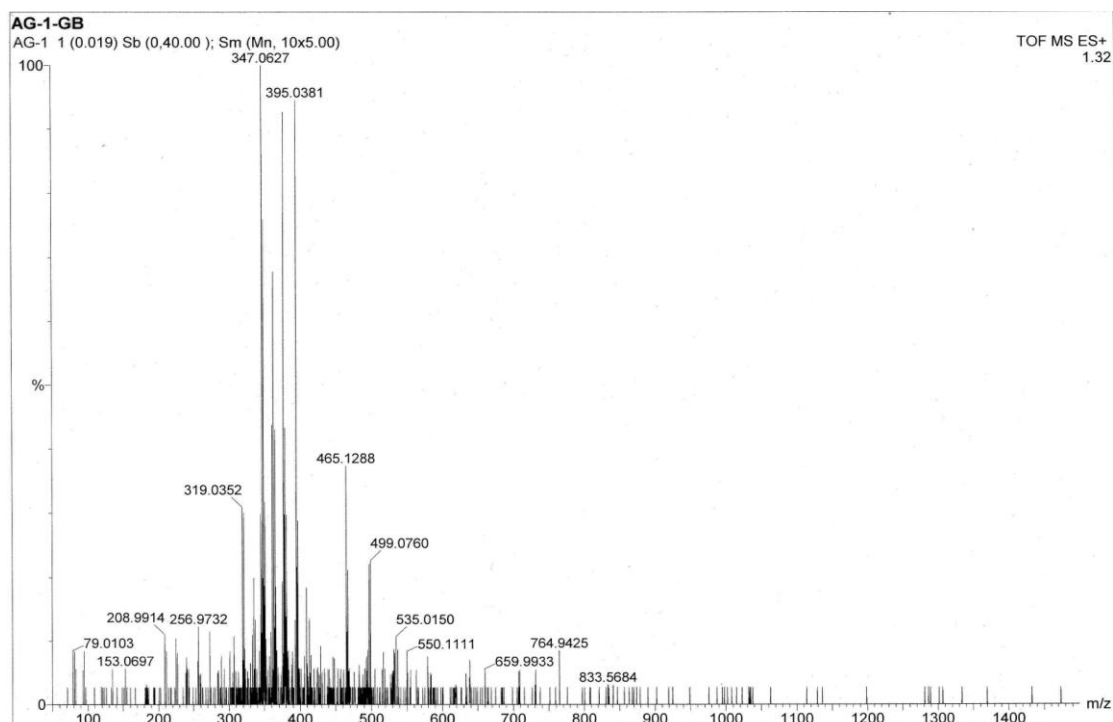


Figure 4: High Resolution Mass spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.



¹H-NMR spectrum of compound in Figure 1 exhibited the presence of 32 protons. Among which $\delta = 5.40$ ppm (1H, dt, $J = 6.8, 0.8$ Hz) and 5.10 ppm (1H, dt, $J = 6.8, 0.2$ Hz) confirmed the presence of two olefinic protons. Two different type of -CH₂- (Methylene) protons was also observed at 2.10 ppm (2H, m), and 2.0 ppm (2H, m). Two -OH protons was located at 4.12 ppm (2H, d, $J = 6.8$ Hz) and another two -OH protons was found at 2.6 ppm (2H,

br s) in the $^1\text{H-NMR}$ - spectrum with remaining others characteristic protons, which supports the proton-skeleton of the isolated compound (Figure- 5).

Figure 5: ^1H NMR spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.

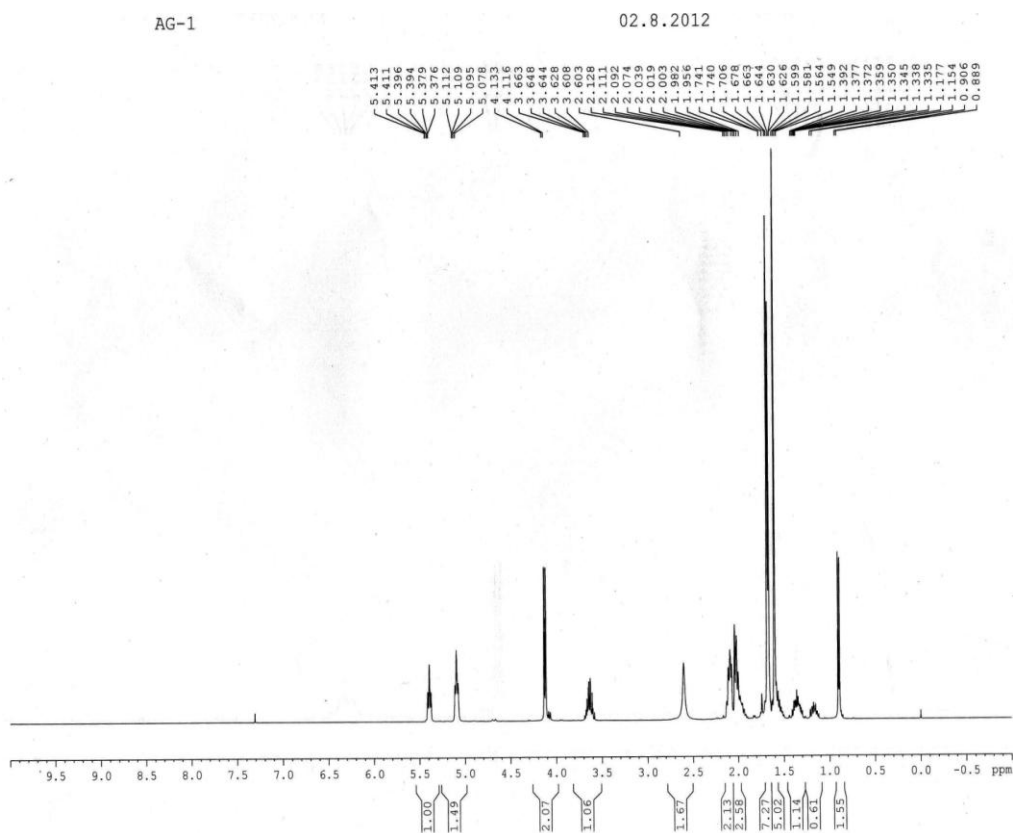
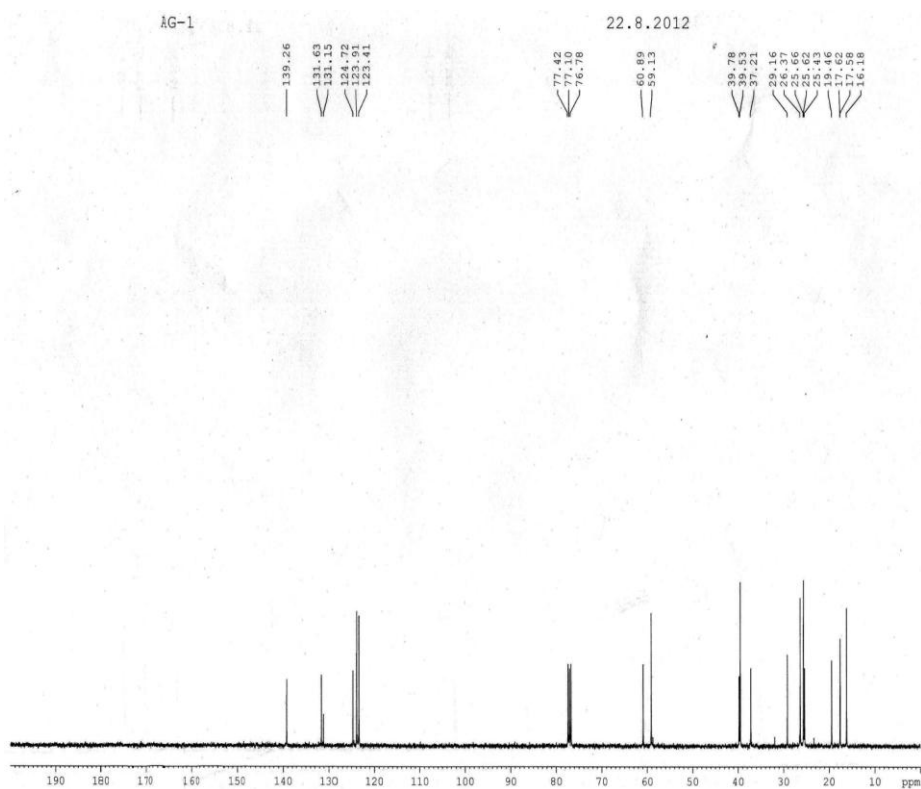
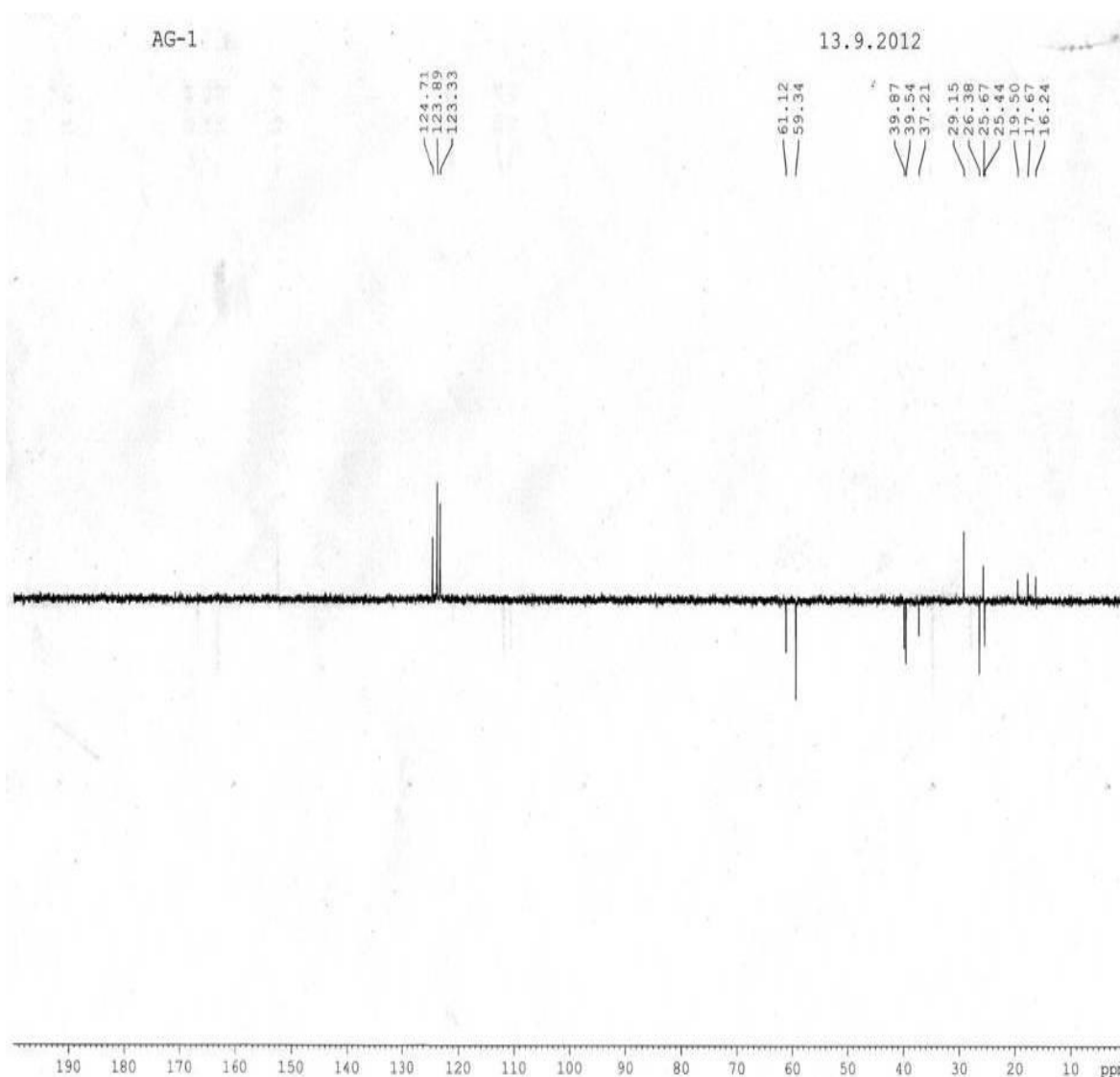


Figure 6: ^{13}C NMR spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.



The ten peaks in ^{13}C -NMR-spectrum clearly indicated the presence of 19 different carbon atoms which confirmed the carbon-skeleton of the above maintained compound (Figure-6).

Figure 7: DEPT- 135 spectroscopy of 2, 3, 4, 8a, 9, 9a-hexamethyl -2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol.



The HRMS- spectrum of the isolated compound was found 347.0627 (M+Na) (Figure- 7). Hence, the molecular formula of the isolated fraction must be $\text{C}_{19}\text{H}_{32}\text{O}_4$ and its structure was shown in Figure 1.

CONCLUSION

The isolated fraction 2,3,4,8a,9,9a-hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol shows antibacterial activities against some major plant pathogenic bacteria. Isolation and characterization of this terpenoid including evaluation of its antibacterial potentiality may also help us to use 2, 3, 4, 8a, 9, 9a -hexamethyl-2,3,3a,4,4a,5,8,8a,9,9a-decahydro-1H-cyclopenta [b] naphthalene-1,2,3a,4a-tetraol as crop protectant.

ACKNOWLEDGEMENTS

This work was supported by Grants in Aid from the DST- PURSE (Grant No. FD/1626, dated: 29.1.2013) and University of Kalyani (Grant No. Rev/ 1124 of 12-13, dated: 28.2.2013), Nadia, West Bengal.

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