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ISOLATION AND CHARACTERISATION OF FLAVONOID FROM *ACHYRANTHES ASPERA LINN.*

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ABSTRACT

In India *Achyranthes aspera* Linn (Amaranthaceae) is commonly used as a phytotherapeutic agent.6-Prenyl Apegenin (Flavonoid) has been isolated from an ethyl acetate extract of the seeds of *Achyranthes aspera* Linn. The structure of the isolated compound was established by modern spectroscopic techniques like UV, ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The extract was found to show mild antibacterial activity.

Keywords: *Achyranthes aspera* Linn, 6-Prenyl Apegenin, Antibacterial activity.

INTRODUCTION

Achyranthes aspera Linn, locally known as Apang. This is distributed throughout India, along the roadsides and waste places as well as on hills up to 900 meters [1]. The height of an enect herb up to 1m.Long cylindrical thick, secondary and terteary roots present [2].

Ayurvedi and Yunani doctors use the stems, leaves and fruits as remedy for piles, renal dropsy, kidney stone, cough, pneumonia, skin eruptions, snakebite, gonorrhea, dysentery, diuretic [3], antisyphilitic [4], contraceptive [5], anti-cancer [6], hypoglycemic [7] etc. Flavonoids thus constitute one of the largest groups of naturally occurring phenols. Flavonoids are phenolic and hence change in colour when treated with base or with ammonia. They are more highly coloured in the acidic medium [8]. In this paper the isolation and cauterization of 6-Prenyl Apegenin and the antibacterial activity of ethyl acetate extract of the seeds of this plant are reported.

MATERIALS AND METHODS

Plant material

The seeds of *A. aspera* (2.5kg) were collected during the month of December 2007 near the campus of Ultra College of Pharmacy, Madurai and authenticated by Dr.D.Steephen, Professor, Department of Botany, The American College, Madurai. A voucher specimen of the sample was deposited in the Department of Pharmaceutical Chemistry for the future reference.

Extraction

The seeds were shade dried and powdered. The seed were extracted with petroleum ether (60-80°C) and 80% v/v ethyl alcohol in soxhlet apparatus by simultaneous extraction for 72 hours. The extracts were concentrated in vacuum. The aqueous alcoholic concentrate on simultaneous extraction with petroleum ether (60 – 80°C), benzene and ethyl acetate. The petroleum ether (5gms), benzene (7gms) and ethyl acetate (12gms) were obtained. The fractions were studied for preliminary phytochemical analysis. The ethyl acetate fraction gave positive reaction with neutral ferric chloride (presence of phenolic compound). So the ethyl acetate fraction was resulted for the isolation of flavonoid compound by column chromatography.

Preliminary Phytochemical Investigation Test for Carbohydrates

Molish Test

To 2-3 ml aqueous of extract, few drops of alpha naphthol solution in alcohol were added and it was shaked well. Then concentrated H_2SO_4 was added through the sides of the test tube.

Test for Reducing Sugars

Fehling's Test

1ml Fehling's A and 1ml Fehling's B solutions were mixed and it was boiled for one minute. Equal

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volume of extract was added to it. It was heated in a boiling water bath for 5-10min.

Benedict's Test

Equal volume of Benedict's reagent and extract was mixed in a test tube. It was heated in boiling water bath for 5 minutes.

Test for Monosaccharides

Barfoed's Test

Equal volume of Barfoed's reagents and extract was mixed and it was heated for 1-2 mins in a boiling water bath and then it was cooled.

TEST for Proteins

Biuret Test

To 3ml of extract test solution, 4% NaOH and few drops of 1%$CuSO_4$ solutions were added.

Millon's Test

3ml of extract was mixed with 5ml Millon's reagent.

Xanthoprotein Test

3ml of the test solution was mixed with 1ml concentrated H_2SO_4. White precipitate was observed. The precipitate was boiled and changed. To this NH_4OH was added and the precipitate turned to orange.

Test for Aminoacids

Ninhydrin Test

3ml of the test solution was heated with 3 drops of 5% ninhydrin solution in a boiling water bath for 10 mins and then the colour change was observed .

Salkowski's Test

To 2ml of extract, 2ml of chloroform and 2ml of concentrated H_2SO_4 were added and it was shaked well and the colour was observed.

Libermann Burchard Reaction

2ml of extract was mixed with chloroform. To this solution 1-2ml acetic anhydride was taken and 2 drops of concentrated H_2SO_4 was added from the sides of test tube and the colour was observed.

Test for Saponin Glycosides

Foam Test

The drug extract or dry powder was shaked vigorously, with water. The foam was observed.

Heamolytic Test

The drug extract or dry powder was added to one drop of blood and it was placed on a glass slide and heamolytic zone was observed.

Test for Glycosides

Test for Cardiac Glycosides

Baljet's Test Glycosides

The extract was mixed with sodium picrate solution and the colour of resulting solution was observed.

Legal's Test

To alcoholic solution extract, 1ml pyridine and 1ml sodium nitro prusside were added and the colour was observed.

Keller-Killani Test

To 2 ml of extract, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added. This solution was carefully transferred to the surface of 2ml concentrated H_2SO_4 and the observation was noted down.

Test for Anthraquinone Glycosides

Borntragar's Test

To 3ml of extract, dilute H_2SO_4 was added and it was boiled and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and it was shaked well. The organic solvent layer was separated and ammonia was added to it and the colour of ammoniacal layer was observed.

Test for Phenolic Compounds

To 2-3ml of the alcoholic solution extract, few drops of following reagents were added.

Ferric Chloride Test

To 2-3ml of alcoholic solution of extract, few drops of 5% w/w ferric chloride solution was added. And the colour change was observed.

Test for Flavonoids

Shinoda's Test

To the extract, 5ml of 95% ethanol was added. Then few drops of concentrated HCl and 0.5gm of magnesium turnings were added and the observation was noted down.

Test for Alkaloids

The extracts were evaporated separately. To the residue, dilute HCl was added and it was shaked well and filtered. With the filtrate, the following tests were performed.

Dragendroff's Test

To 2-3ml of filtrate, few drops of Dragendroff's reagent was added and precipitate was observed.

Mayer's Test

To 2-3ml of the filtrate, few drop of Mayer's reagent was added and the precipitate was observed.

Hager's Test

To 2-3ml of the filtrate Hager's reagent was added and the precipitate was observed.

Wagner's Test

To 2-3ml of the filtrate, few drops of Wagner's reagent was added and the precipitate was observed [9,10].

Isolation of Flavonoid

The ethyl acetate extract concentrate (10gms) of *A. aspera* was chromatographed in silica gel (60-120mesh, Merck, India 300gms, 110cm x 3cm) column built in benzene. Elution of column (gradient elution) with benzene and ethyl acetate mixtures of increasing polarity. The fraction of 100ml was collected each time. The fraction was monitored using TLC (silica gel G Merck, India). TLC identified by iodine chamber. The details of fractions are given in Table No.1

The 161-219 fractions of (20:80) benzene : ethyl acetate were combined. They were gave double spot on TLC and rechromatographed. The rechromatographic

column (60-120 mesh, Merk, India 50gms, 30cm x 1.5cm) built with chloroform and eluted with chloroform and methanol (9:1) mixtrure. The fractions of 10ml was collected each time. The fractions eluted from 29-39 yielded a single compound. (on testing by TLC) and responded positively for ferric chloride test (characteristic of phenolic compound). The details of fractions are given in Table No.2. This compound was taken up for spectral characterization. The running properties of the isolated compound are given in Table No.3. The UV, ^{1}H NMR, ^{13}C NMR, DEPT 90, DEPT 135 and ESI-MS were recorded for the isolated compound.

Anti-bacterial activity

The antibacterial activity of extract of the seeds of *Achyranthes aspera* Linn was determined against *E.coli*, *Bacillus substilis*, *Stapylococcus Epidermitis*, and *Staphylo coccus areus*. For the determination of antibacterial activity the cup plate method was employed. Nutrient Agar was used as a basal medium for test bacteria [12].

RESULTS AND DISCUSSION

Physical and chemical method

In column chromatography a single compound was isolated in the fraction of 29-39 in recolumn (Chloroform : Methanol , 9:1) from the 20:80 benzene, ethyl acetate fraction of (161-219) ethyl acetate extract residue. It was designated as isolated compound, slight yellow amorphous powder. Melting point 231 ± 1^{o}C, gave positive reaction to ferric chloride test showing (Dark brown colour) typical colour for flavonoid.

Ultra Violet Spectrum

Isolated compound (M.P.231± 1^{o}C) showed an intense λ_{max} at 325 nm (band I) and at 225 nm (band II) indicative of the flavone nature of it. A bathochromic shift of band I (+45nm) on addition of NaOMe suggested the presence of hydroxyl at C-4'. A bathochromic shift of band II (+ 15nm) on addition of NaOAc indicated the presence of hydroxyl group at C-7. The absence of NaOAc / H_3BO_3 shift in band I confirmed the absence of ortho dihydroxy group in ring B. No change in band I on addition of $AlCl_3$ /HCl, which indicated the presence of 5-OH along with 6-prenyl group. Thus the UV spectral data with diagnostic reagent suggested the likely presence of substituted flavone with hydroxyls at 5,7 and 4'positions [13].

^{1}H NMR Spectrum

The presence of a signal at δ 12.90 ppm in ^{1}H NMR spectrum also confirmed the presence of hydroxyl group at C-5 (Table No. 6). In the 500 MHz ^{1}H NMR spectrum of isolated compounds the signal at δ 9.40 ppm account for the 4'OH group. The doublet at δ 6.36 ppm was assigned for H-8. The doublets at δ 7.91 and δ 6.95 ppm were assigned for H-2',6' and H- 3',5' respectively. The unoxygenated C-3 proton showed up at δ 6.28 ppm. The presence of down field shift signal of six proton at δ 1.20 (6H, H-4λ and H-5λ),3.11 (H-1λ) and δ 5.32 ppm indicated the presence of prenyl group in isolated compound.

^{13}C NMR Spectrum

The ^{13}C NMR spectrum of isolated compound showed the presence 20 assignable carbon signals. The ^{13}C NMR, DEPT 90, DEPT 135 studies revealed the presence of 10 quaternary carbons, 7 methine signals, one methylene signals and two methyl signals in the isolated compound. From the ^{13}C NMR, DEPT 90, DEPT 135 data, we assigned the signals at δ 102.421, 94.421, 128.642, 127.919, 166.909, 166.909, 122.535, 28.365, 23.289, 18.830 ppm as quaternary carbon. The signals occurring at δ 23.28 and 18.83 ppm were assigned to the methyl carbons of prenyl substituent. The presence of one methylene signal at δ 28.36 ppm was confirmed from the DEPT 135 spectra. The signals at δ 102.879, 94.421, 128.642, 127.919, 116.909, 116.909, 122.535 ppm were assigned as the methine carbons of isolated compound. The UV spectra with $AlCl_3$ and HCl shift reagent indicated the presence of prenyl substituent in the isolated compound. The absences of H-6 signal in ^{1}H NMR also confirmed the substituted nature of the C-6. The carbon signals at δ 28.365 ppm (C-1λ), 122.535 ppm (C-2λ), 122.535 ppm (C-3λ), 23.289 ppm (C-4λ) and 18.830 ppm (C-5λ) confirmed the presence of prenyl substituent in the isolated compound [15].

Mass Spectrum

ESI-MS spectrum of isolated compound exhibited a peak (M^{+} + 1$^{\bullet}$) at *m/z* 339. A fragment peak at *m/z* 270 and *m/z* 271 accounting for apigenin. A fragment ion at *m/z* 153 consistent with the retro – Diels Alder fragmentation showed the compound with 5,7 dihydroxy flavone skeleton. A fragmentation at *m/z* 121 confirmed the presence of hydroxyl group at C-4'in ring B. Its molecular formula C_{20} H_{18} O_5 molecular weight 338 was determined by ESI-MS measurement. Based on the R_f values UV, ^{1}H NMR, ^{13}C NMR, DEPT 90, DEPT 135 and ESI-MS spectral studies the isolated compound has been characterized as ***6 prenyl apigenin (or) 5,7,4–trihydroxy 6-prenyl flavone*** [16].

Table 1. Chromatographic Fractionation of *A.aspera*

Fractions collected	Eluent composition	Remarks
1-66	100% benzene, 90/10 to 70/30 benzene/ethyl acetate	Light greenish yellow coloured mixture of compound.
67-97	60/40 to 40 / 60benzene / ethyl acetate	greenish yellow colour unseparable mixture
98-144	30/70 benzene / ethyl acetate	Yellow colour unseparable mixture.
145-160	20/80 benzene / ethyl acetate	Light yellow colour unseparable mixture.
161-219	20/80 benzene / ethyl acetate	Light yellow colour mixture of two compounds.

Table 2. Rechromatographic Fractionation of *A.aspera*

Fractions collected	Eluent composition	Remarks
1-29	90/10 Chloroform / methanol	Yellow colour mixture compound
29-39	90/10 Chloroform / methanol	Yellow colour single compound.

Table 3. R_f (x100) Values of Isolated Compound

S.No	Solvent system	R_f (x100) Value
1	$CHCl_3$:MeOH	23.52
2	EtOAc:C_6H_6	15.38
3	EtOAc: $CHCl_3$	19.60

Table 4. Anti-Bacterial Activity of Ethyl Acetate Extract

Organism	Ethyl Acetate Extract		Standard (Benzyl penicillin)	
	A	B	A	B
	0.05ml	0.1ml	0.05ml	0.1ml
E.Coli	16	19	25	27
B.Substilis	15	16	28	33
S.Epidermidis	19	19	31	32
S.Aureus	13	14	28	31

The result of anti-bacterial activity proved that compound A and B showed anti-bacterial activity when compared with standard drug Benzyl penicillin.

Figure 1. Ultra Violet Spectrum of isolated compound

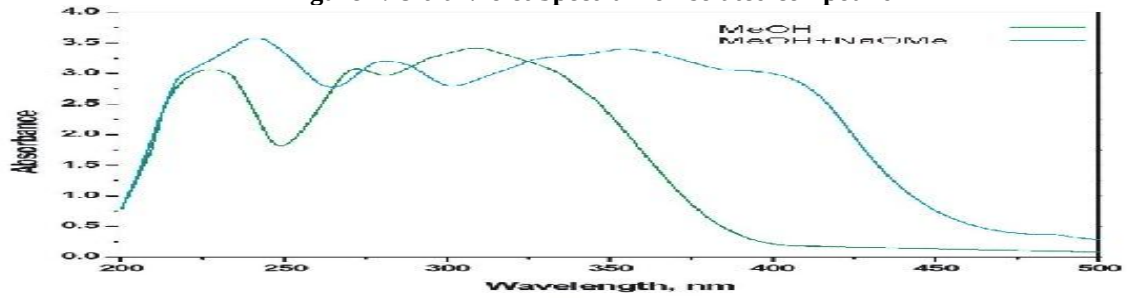


Figure.3

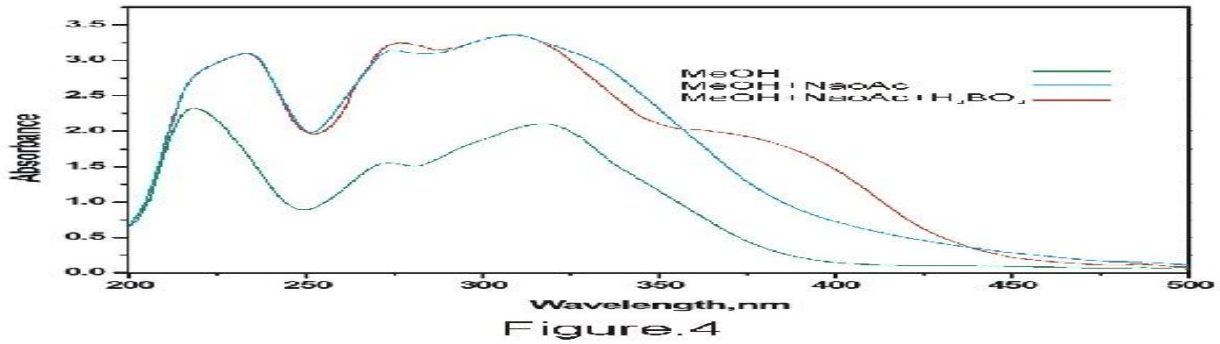


Figure.4

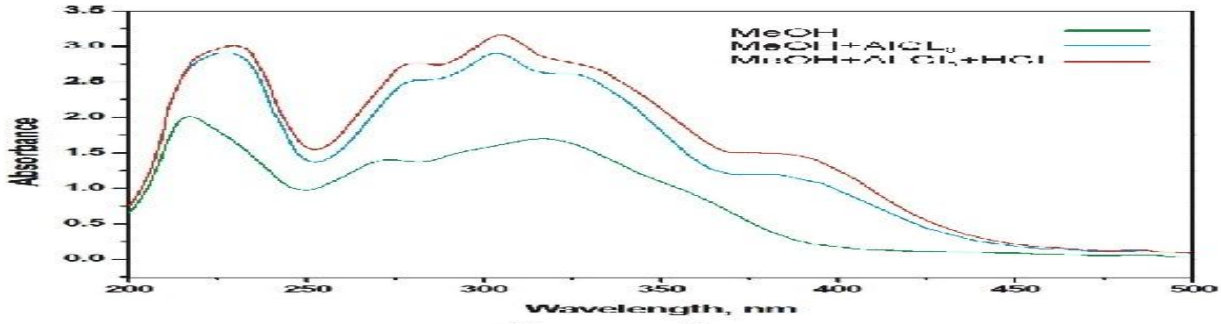
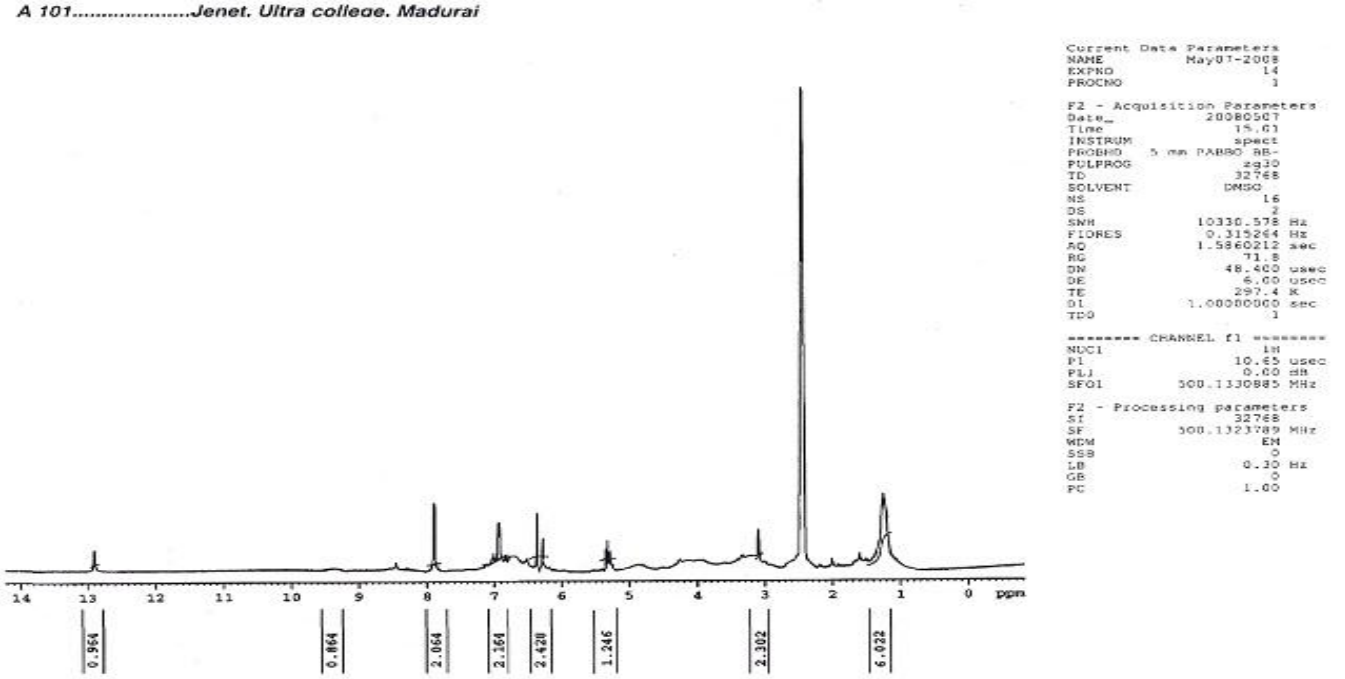


Figure.5

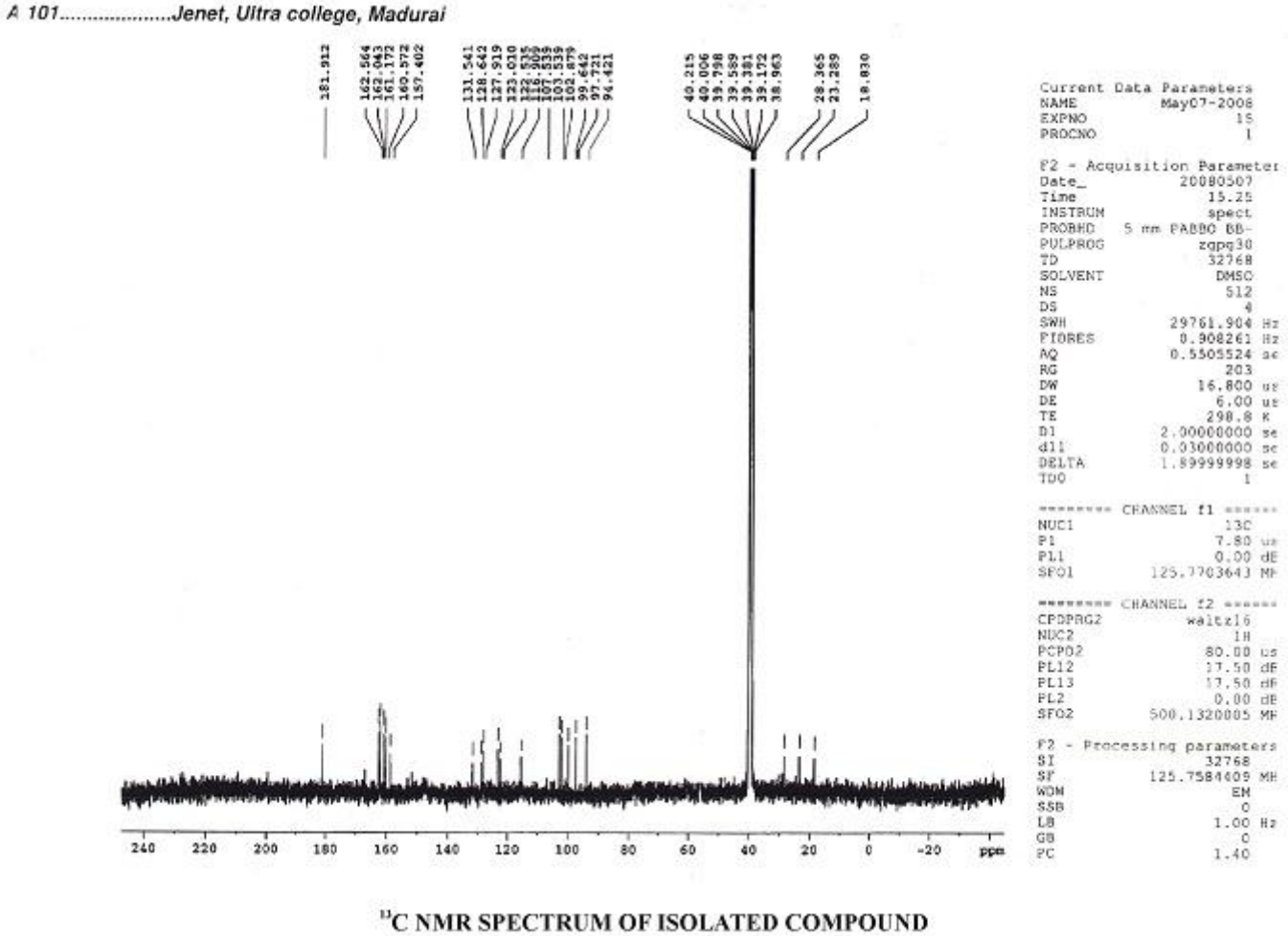
UV SPECTRUM OF ISOLATED COMPOUND

Figure 2. ^{1}H NMR Spectrum of isolated compound



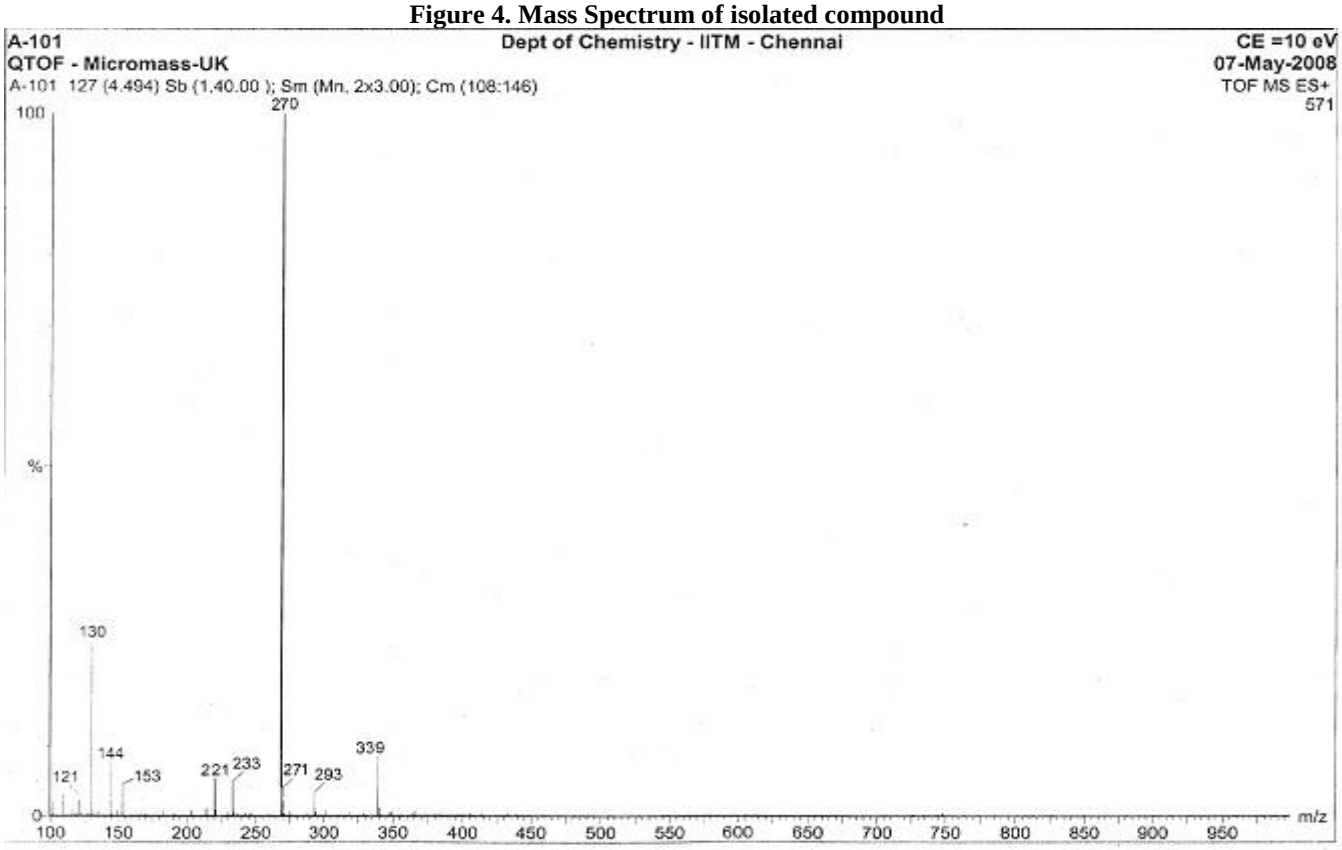
^{1}H NMR SPECTRUM OF ISOLATED COMPOUND

Figure 3. ^{13}C NMR spectrum of isolated compound



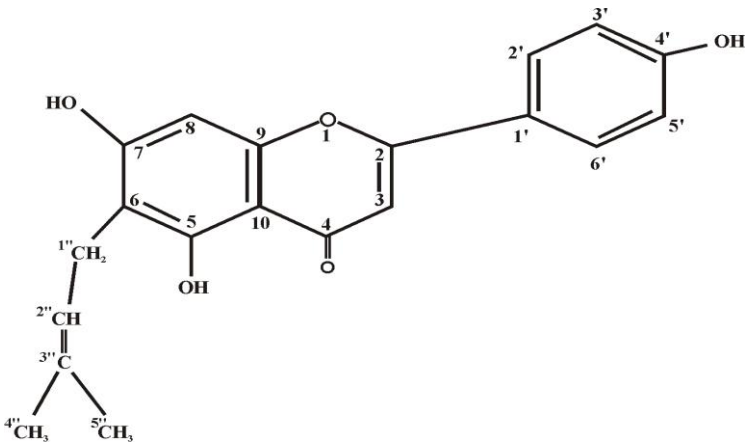
^{13}C NMR SPECTRUM OF ISOLATED COMPOUND

Figure 4. Mass Spectrum of isolated compound



MASS SPECTRUM OF ISOLATED COMPOUND

The structure of isolated compound has been given be



CONCLUSION

The Shade-dried seeds of *A.aspera* (Amaranthaceae) were simultaneously extracted with petroleum ether and 80% ethyl alcohol. The concentrate of 80% ethyl alcohol extract, fractioned with benzene and ethyl acetate. The preliminary phytochemical investigation has been shown the presence of flavonoid in ethyl acetate and ethyl alcohol extracts. The residue of ethyl acetate fraction on column chromatography yielded on isolated compound. The isolated compound gave positive test (ferric chloride and shinoda's test) for phenolic compound.

The isolated compound, M.P 231 ± 1^{o}C, a slight yellow amorphous powder on the UV studies indicated a flavone skeleton with free hydroxyl groups at 5, 7 and 4' positions. The ^{1}H NMR studies of isolated compound revealed the presence of prenyl group at C_6 position of flavone skeleton. The ^{13}C NMR data helpful in the identification of prenyl and also confirmed the position of attachment of prenyl moiety. The ^{13}C NMR spectrum of isolated compound showed the presence of 20 assignable carbon signals. The ^{13}C NMR, DEPT 90, DEPT 135 studies revealed the presence of 10 quaternary carbons, 7

methine signals, one methylene signal and two methyl signals in the isolated compound.

Finally the mass spectrum confirms the molecular weight and prenyl substitution of the isolated compound. Based on the R_f, UV, ^{1}H NMR, ^{13}C NMR, DEPT- 90, DEPT- 135 and ESI-MS, studies the structure of isolated compound has been characterized as *6 prenyl apigenin*. This is the first report of a flavonoid in this plant.Theory and detailed experimental work involved in the evaluation of anti-bacterial studies on ethyl acetate extract of *Achyranthes aspera* Linn. From the result it is inferred that *Achyranthes aspera* Linn have anti-bacterial activity.

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