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Research Article

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Preliminary Phytochemical Evaluation, Isolation and Spectroscopic Characterization of Constituents in the Dried Extracts of the Whole Plant *Crotalaria Biflora* (L)

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Abstract: Several drugs currently available today for a variety of ailments are developed directly or indirectly from plant source. With this view, the plant *Crotalaria biflora* was selected for our research and initially it was decided to evaluate the phytochemical compositions and their characterization by different spectral methods. The whole plant *Crotalaria biflora* was collected, authenticated, dried in shade and powdered by mechanical grinder. The powdered material was extracted individually with solvents viz., petroleum ether, chloroform, ethyl acetate and methanol in soxhlet apparatus. The dried extracts thus obtained were employed for the preliminary phytochemical evaluation for the presence of alkaloids, glycosides, tannins, flavonoids, terpenoids, sterols, saponins etc., followed by column chromatography with the solvents of ascending order of polarity from hexane to ethyl acetate in the ratio from 95:5 to 0:100 and thin-layer chromatography separation by eluted with different ratio of hexane:ethyl acetate like 90:10, 75:25, 50:50 and 25:75 and detected with vanillin in sulphuric acid. Based on the yield obtained in the chromatographic separation, the compounds selected were subjected to different spectroscopic evaluation such as FTIR, ^{13}C NMR, ^{1}H NMR and mass spectroscopic methods. In the preliminary phytochemical evaluation, significant positive results were obtained from the tests for alkaloids, terpenoids and sterols. In column chromatography, 900 fractions were collected and each fraction was subjected to TLC separation and five pure compounds were isolated with the help of TLC. Based on the yield, two compounds were selected for spectral evaluation and the compound 1 was identified as (Z)-(6-hydroxy-1-methyl-5-oxo-1,2,5,6,7,8-hexahydroazocin-4-yl) methyl acetate and the compound 2 was identified as β-Sitosterol. These results are useful for further investigation in the future particularly the biological activities of these extracts.

Keywords: *Crotalaria biflora*, preliminary phytochemical evaluation, column chromatography, thin-layer chromatography, spectroscopic evaluation.

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1. INTRODUCTION

Since ancient times, cultures all over the world have used plant sources for therapeutic purposes. Traditional medicinal knowledge, also known as indigenous or folk medicinal system is developed by various societies of the world over generations and widely in practice even today [1]. Several common drugs in usage today are developed from the plants used in traditional medicinal systems [2-5]. Medicinal plants are generally found throughout the world, particularly they are abundant in tropical countries [6]. Several phytochemicals such as alkaloids, glycosides, flavonoids, tannins, phenolic compounds etc., are present in the different parts of the plants like root, stem, flower, fruit and exudates can be used to treat different types of chronic as well as infectious diseases[7]. Plants used in the traditional healing system requires a systematic evaluation that may give more promising data about their medicinal value and may be useful to meet the rising demand of novel agents to combat the infections and diseases[8]. With this view, it was decided to conduct a study on the plant with ethnomedicinal value, thus, the plant *Crotalaria biflora* was selected for the present study. The genus *Crotalaria* belongs to the Fabaceae family which consists of about 500 species distributed all over the world including African countries. Several species of *Crotalaria* are cultivated as crops and consumed by the rural population for various purposes such as medicine, food, green manure, fodder etc., throughout the world[9]. In India, *Crotalaria* plants are commonly found in the fields and forest lands throughout the year and sometimes cultivated. The plant *Crotalaria biflora*, commonly found in south India at an altitude up to 300m mean sea level is not scientifically explored previously to our knowledge was selected for the present study. The seed of this plant is edible one by rural population because of its protein content[1]. Now, the present study focused on preliminary phytochemical screening, isolation and characterization of chemical constituents in the whole plant *Crotalaria biflora*, an attempt to provide a direction for further research.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant

The whole plant of *Crotalaria biflora* was collected from the Mekkarai, the village located near the foothills of Western Ghats, Tirunelveli District, Tamil Nadu, India. The collected plant was identified and authenticated by Dr Chelladurai, Research Officer-Botany (Scientist–C), Central Council for Research in Ayurveda & Siddha, Govt. of India.

2.2 Preparation of Powdered Plant Material

By using the mechanical grinder, a coarse powder was prepared from the whole plant material suitably dried in the shade for about ten days. The prepared powder was stored in the airtight container for extraction and further evaluation.

2.3 Extraction of Powdered Plant Material

Extraction was done successively with solvents of ascending order of polarity viz., petroleum ether, chloroform, ethyl acetate and methanol in the soxhlet apparatus assembly. For extraction, about 30g of powdered plant material was moistened with the respective solvent, packed in the soxhlet extractor and extracted with 500ml of respective solvents individually. After each extraction, by using the same dried marc, subsequent extraction was done. Each extract was filtered, distilled and the dried extract was collected and the percentage yield of each was noted and preserved for preliminary phytochemical screening.

2.4 Preliminary Phytochemical Screening

Preliminary phytochemical screening was done in reference to the previous literature [10-15].

2.4.1 Test for Alkaloids

A small quantity of dried extract and alcohol were mixed and acidified with dilute hydrochloric acid and filtered. The filtrate was used to detect the presence of alkaloids by the following tests.

a. Mayer's Test
2ml of the filtrate was shaken with an equal quantity of Mayer's reagent and observed for the formation of creamy precipitate.

b. Wagner's Test
2ml of the filtrate was mixed with an equal quantity of Wagner's reagent and observed for the formation of a reddish-brown precipitate

c. Hager's Test
1ml of Hager's reagent was added to the 2ml of filtrate and observed for the formation of yellow precipitate.

d. Dragendorff's Test
2ml of the filtrate was treated with an equal quantity of Dragendorff's reagent and observed for the formation of the orange-red precipitate.

2.4.2 Tests for Glycosides

A pinch of dried extract and dilute hydrochloric acid were mixed and kept in the water bath for one hour. The hydrolysate so obtained was employed for the chemical test for glycosides.

a. Legal Test
2ml of hydrolysate was treated with an equal quantity of pyridine and freshly prepared sodium nitroprusside solution. This mixture was alkalinized with few drops of 20% sodium hydroxide solution and observed for the formation of deep red colour.

b. Baljet Test
2ml of hydrolysate was treated with an equal quantity of sodium picrate reagent and observed for the formation of a yellow to orange colour.

c. Borntrager's Test
The residue obtained from the evaporation of hydrolysate was mixed with water and shaken with an equal volume of chloroform. The chloroform layer was separated and shaken well with an equal volume of dilute ammonia solution and observed for the formation of pink colour in the ammonical layer.

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d. Modified Borntrager's Test

The residue obtained from the evaporation of hydrolysate was treated with little quantity of ferric chloride and dilute hydrochloric acid. This mixture was shaken with an equal volume of chloroform. The chloroform layer was separated and shaken well with an equal volume of dilute ammonia solution and observed for the formation of pink colour in the ammonical layer.

2.4.3 Test for Phenolic compounds and Tannins

a. Ferric chloride Test
Two to three drops of dried extract in water was treated with a 5% dilute ferric chloride solution and observed for the formation of blue colour.

b. Gelatin Test
Two to three drops of dried extract were mixed with water and filtered. To the filtrate 2% gelatin containing 10%, sodium chloride solution was added and observed for the formation of milky white precipitate.

c. Lead acetate Test
Two three drops of dried extract in water was treated with a 10% lead acetate solution and observed for the formation of bulky white precipitate.

d. De-colourization Test
Two to three drops of an aqueous solution of dried extract was treated with dilute potassium permanganate solution and observed for the decolourization of potassium permanganate.

2.4.4 Tests for Flavanones and Flavonoids

a. Aqueous Sodium hydroxide Test
A pinch of dried extract was treated with aqueous sodium hydroxide solution and observed for the formation of yellow colour.

b. Ammonia Test
Filter paper wetted with alcoholic solution of dried extract was exposed to ammonia vapour and observed for the formation of yellow colour.

c. Shinoda Test
An alcoholic solution of dried extract was treated with little quantity of magnesium or zinc and dilute hydrochloric acid was added to it and observed for the formation of orange-red or violet colour.

2.4.5 Test for Carbohydrates

A pinch of the dried extract was mixed with water and filtered. The filtrate was used in the following tests to detect the presence of carbohydrates.

a. Molisch's Test
2ml of the filtrate was mixed with equal quantity of Molisch's reagent and concentrated sulphuric acid was added through the sides of the test tube without shaking and observed for the formation of the violet ring at the junction of the two solutions.

b. Fehling's Test
1 ml of the filtrate was treated with an equal quantity of Fehling solution A and B and kept in the water bath for 30minutes and observed for the formation of reddish colourat the base of the test tube.

c. Benedict's Test
2ml of the filtrate was treated with an equal quantity of Benedict's reagent and kept in the water bath and observed for the formation of a reddish precipitate.

2.4.6 Tests for Proteins and Amino acids

a. Millon's Test
A small quantity of dried extract was treated with 2ml of Millon's reagent and observed for the formation of white precipitate, on warming, it turns into a red colour solution.

b. Biuret Test
A small quantity of dried extract was treated with a few drops of 2% copper sulphate solution. To this excess potassium hydroxide solution was added and observed for the formation of violet colour.

c. Ninhydrin Test
A small quantity of dried extract was treated with ninhydrin reagent and kept in the water bath and observed for the development of violet colour.

2.4.7 Test for Terpenoids

a. Salkowski's Test
A small quantity of dried extract was mixed with chloroform. To this mixture, equal quantity of concentrated sulphuric acid was added. Formation of red colour in the chloroform layer and greenish-yellow fluorescence in the acid layer indicates positive result.

2.4.8 Test for Sterols

A small quantity of the dried extract was mixed with alcohol and refluxed with alcoholic potassium hydroxide solution for saponification. The saponified mixture was diluted and extracted with solvent ether. The ethereal extract was evaporated and the residue obtained was used for the detection of sterols.

a. Liebermann-Burchard Test
A small quantity of the residue, 2ml of chloroform and acetic anhydride was added and concentrated sulphuric acid was added to it at the side walls of the test tube. Formation of green colour in the upper portion which changes to blue or violet colour indicates positive result.

b. Salkowski's Test
A small quantity of residue was treated with 2ml of chloroform and concentrated sulphuric acid was added to this mixture and observed for the formation of red colour in the lower layer.

2.4.9 Test for Saponins

a. Foam Test (Froth test)
A small quantity of dried extract was mixed with 20ml of distilled water and shaken for 15minutes and observed for the formation of foam.

b. Haemolysis Test

A drop of blood was taken in a slide and a little quantity of dried extract was mixed to it and observed for haemolysis.

2.4.10 Test for Gum and Mucilage

25ml of absolute alcohol was added to the 10ml of the aqueous extract with constant stirring. Filtered and the precipitate formed was dried in air and examined for swelling properties.

2.4.11 Test for Volatile oil

50gm of powdered material of the whole plant was subjected to hydrodistillation by using Clevenger apparatus. The distillate was collected and observed for the presence of an oil layer.

2.5 Fractionation of Crude Extract by Column Chromatography

10 gm of the dried extract was mixed with 20gm of Silica gel (60-120 mesh) and sufficient quantity of hexane and the admixture was packed in the column of 2.4dia, with silica gel and eluted with solvents with ascending order of polarity from hexane to ethyl acetate in the ratio from 95:5 to 0:100. The fractions collected were subjected to thin-layer chromatography (TLC) separation. The fractions showed the same R*f* value were combined and concentrated by using the rotary evaporator.

2.6 Thin-layer Chromatography (TLC)

An aliquot of all concentrated fractions was loaded on the activated silica gel TLC plates (20×20cm) and eluted with different ratios of hexane: ethyl acetate like 90:10, 75:25, 50:50 and 25:75. Spots were identified by using vanillin in the sulphuric acid reagent. Isolated compounds were characterized by spectroscopic evaluation.

2.7 Characterization by Spectroscopic Methods

2.7.1 ^{13}C NMR

^{13}C NMR spectral evaluation of the isolated compounds was done by using Bruker Avance 100 MHz NMR instrument. About 40 mg of the isolated compound was dissolved in $CDCl_3$: DMSO-d_6, 90:10 in an NMR tube. All the spectra obtained were corrected with equivalent to the solvent signal.

2.7.2 ^{1}H NMR Spectrum

Proton NMR (300 MHZ) spectra of the isolated compounds were recorded in $CDCl_3$. Chemical shifts were noted in PPM (parts per million) downfield in reference with TMS (tetramethylsilane) on the instrument Bruker Ultrashield 400.

2.8 Mass Spectra

Mass Spectra of the isolated compounds were recorded by TOF MS ES Mass Spectroscopy using electron impact process.

2.9 IR Spectrum

IR spectra of the isolated compounds were recorded by using KBr pellets in the range of 4000 – 500 cm^{-1} on FT/IR - 6600, Jasco to elucidate the structure of the compounds.

3 RESULTS AND DISCUSSION

In the present study, the whole plant *Crotalaria biflora* was collected (Figure 1), dried and made into a coarse powder. Extraction was done and dried extract of different solvents such as Petroleum ether, Chloroform, Ethyl acetate and Methanol was collected and their percentage yield was calculated. It was identified that the petroleum ether extract gave 5.3gm, the chloroform extract gave 3.23gm, ethyl acetate and methanol extract gave 3.50 and 8.4gm of dried extract respectively.

Fig 1: *Crotalaria biflora* plant

In the preliminary phytochemical evaluation, the methanol extract showed significant positive results in the tests for alkaloids. In case of tests for glycosides, chloroform, ethyl acetate and methanol extracts indicated its presence. The methanol extract indicated the presence in the tests for phenolic compounds. Ethyl acetate and methanol extracts indicated the presence in the tests for flavonoids, carbohydrates and saponins and gave a significant positive result in the tests for terpenoids. Petroleum ether and chloroform extracts indicated the presence in the tests for sterols. Tests for proteins and amino acids, gum and mucilage and volatile oil gave negative results (Table 1).

Table 1: Preliminary phytochemical evaluation of the dried extract of whole plant *Crotalaria biflora*

S. No.	Chemical Test	I	II	III	IV
1	**Alkaloids**				
a	Mayer's test	-	-	-	++
b	Wagner's test	-	-	-	++
c	Hager's test	-	-	-	++
d	Dragendorff's test	-	-	-	++
2	**Glycosides**				
a	Legal's test	-	+	+	-
b	Baljet's test	-	+	+	+
d	Borntrager's test	-	-	-	-
e	Modified Borntrager's test	+	-	-	-
3	**Phenolic compounds**				
a	Ferric chloride test	-	-	-	+
b	Lead acetate test	-	-	-	+
c	Gelatin test	-	-	-	+
4	**Flavanones and flavonoids**				
a	Aqueous NaoH test	-	-	+	+
b	Ammonia test	-	-	+	+
c	Shinoda test	-	-	+	+
5	**Carbohydrates**				
a	Molisch's test	-	-	+	+
b	Fehling's test	-	-	+	+
c	Benedict's test	-	-	+	+
6	**Proteins and Amino acids**				
a	Millon's test	-	-	-	-
b	Biuret test	-	-	-	-
c	Ninhydrin test	-	-	-	-
7	**Terpenoids**				
a	Salkowski's test	-	-	++	++
8	**Sterols**				
a	Libermann-Burchard's test	+	+	-	-
b	Salkowski's test	++	++	-	-
9	**Saponins**				
a	Foams test/froth test	-	-	+	+
b	Haemolysis test	-	-	+	+
10	**Gum & mucilage**	-	-	-	-
11	**Volatile oil**	-	-	-	-

I – Petroleum ether extract; II – Chloroform extract; III – Ethyl acetate extract; IV – Methanol extract; (+) – Positive result; (++) – Significant positive result; (-) – Negative result

In column chromatography, 900 fractions were collected and each fraction was subjected to TLC separation. Five pure compounds were isolated with the help of TLC (Table 2 & Figure 2).

Table 2: TLC separation of isolated fractions

S. No	No. of fractions	% of solvent	Volume of solvent used in column fractionation (ml)	TLC spot
1	1 - 70	100% Hexane	500	
2	71 - 110	95% Hexane : 5% Ethyl acetate	1000	Compound 1
3	110 - 180	90% Hexane : 10% Ethyl acetate	1000	Compound 2
4	181 - 240	85% Hexane : 15% Ethyl acetate	700	
5	241 - 300	80% Hexane : 20% Ethyl acetate	800	Compound 3
6	301 - 370	75% Hexane : 25% Ethyl acetate	800	Compound 4
7	371 - 430	70% Hexane : 30% Ethyl acetate	800	
8	431 - 600	50% Hexane : 50% Ethyl acetate	1000	Compound 5
9	601 - 650	100% Ethyl acetate	200	

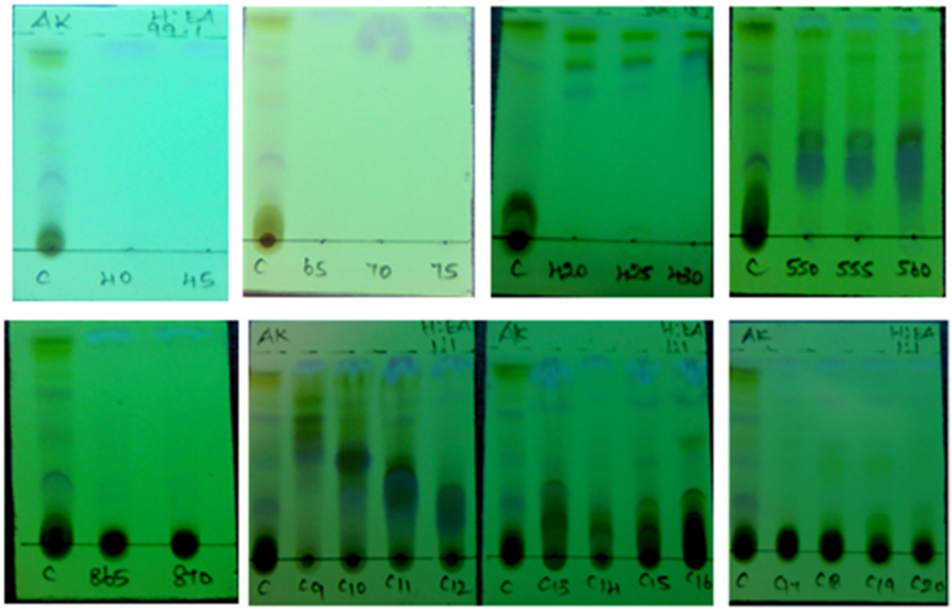


Fig 2: TLC plates of some isolated fractions

Among 5 compounds, 2 were selected for spectral evaluation based on the yield obtained. The selected compounds were analyzed by FTIR, ^{13}C NMR, ^{1}H NMR and Mass spectroscopic evaluation. From the evaluation, compound 1 was identified as (Z)-(6-hydroxy-1-methyl-5-oxo-1,2,5,6,7,8-hexahydroazocin-4-yl) methyl acetate and compound 2 was identified as β-Sitosterol (Figure 3).

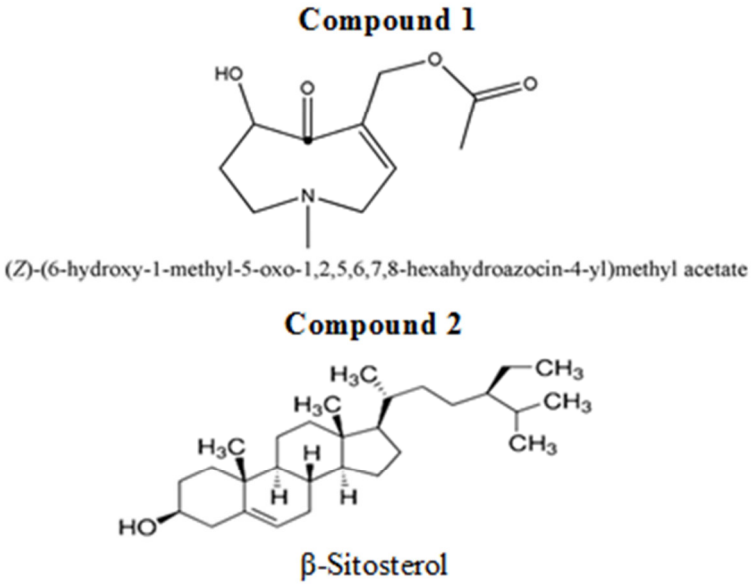


Fig 3: Compounds identified by the spectroscopic evaluation

The ^{13}C NMR of the compound 1, (Z)-(6-hydroxy-1-methyl-5-oxo-1,2,5,6,7,8-hexahydroazocin-4-yl) methyl acetate showed 24.04(methyl), 25.53-49.68(all cyclic CH2), 55.89-60.47(alcohol), 129.06, 135.70(double bond), 171.31(ester), 198.25(N attached to Carbon) and ^{1}H NMR showed 1.00 (methyl), 1.1-2.45(all CH_2), 4.17(ester CH_2), 5.93(N-methyl), 7.29(double bond). In case of the compound 2, β-Sitosterol, the ^{13}C NMR showed 14.00-28.26(CH_3), 28.5-31.90(CH_2), 32.77-39.77(CH), 40.54-51.25 (quarternary carbons), 77.36(C-OH), 121.75-140.75(C=C) and ^{1}H NMR showed 0.71-1.47(methyl protons), 1.52-1.59 (methylene protons), 3.53(CH), 4.12, 5.38(C=CH). The ^{13}C NMR and ^{1}H NMR reports of compound 1 and 2 are shown in Figure 4 & 5. Mass spectra and FTIR reports of compound 1 and 2 are shown in Figure 6 & 7. In recent days, plant based substances gains greater attraction because of their diverse applications and wide awareness about the adverse effects of modern drugs [16]. In the preliminary phytochemical evaluation of the extracts, it was able to identify the presence of alkaloids, terpenoids, flavonoids and sterols. The significant presence of alkaloids in the methanol extracts is in accordance with the previous literature[11,13,14,17]. It was reported that the alkaloids interfere with the cell division process, thus possess the anticancer property[12]. In the present study, the methanol and ethyl acetate extracts showed a strong positive result in the test for terpenoids. This agreed with the previous findings[13,18]. Terpenoids are well known for the anti-microbial, anti-allergic, anti-inflammatroy anti-hyperglycemic, anti-spasmodic and immunomodulatory properties [15]. Likewise, the petroleum ether and chloroform extracts indicated the presence of sterols. Cardiotonic, insecticidal and anti-microbial properties of sterols was documented in the previous studies. The presence of flavones and flavonoids identified in

the ethyl acetate and methanol extract are well documented about their anti-oxidant and anti-cancer activity and lower the risk of heart disease. Several reports indicated their anti-inflammatory, anti-microbial and anti-diabetic properties also [19]. The ethyl acetate and methanol extracts showed the significant presence of phytochemicals. It may be due to their high polarity [13]. TLC profiling of crude extracts is crucial one for the development of quality parameters [20]. In this study, five pure compounds were isolated by TLC and two among them were characterized by spectral studies. Outcome of this study would provide a base for further research which may give more valuable results.

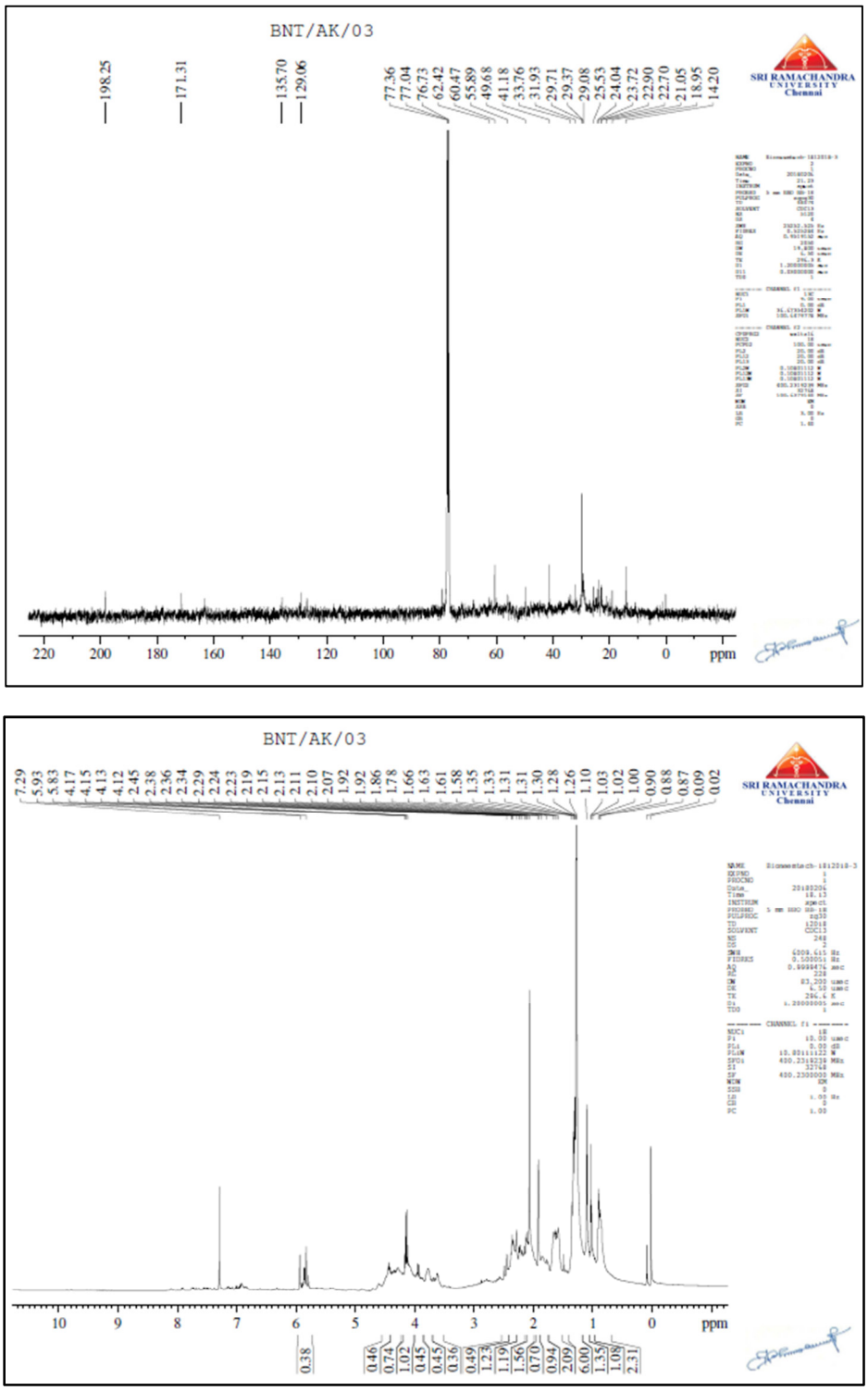


Fig 4: ^{13}C NMR of compound I (A), ^{1}H NMR of compound I (B)

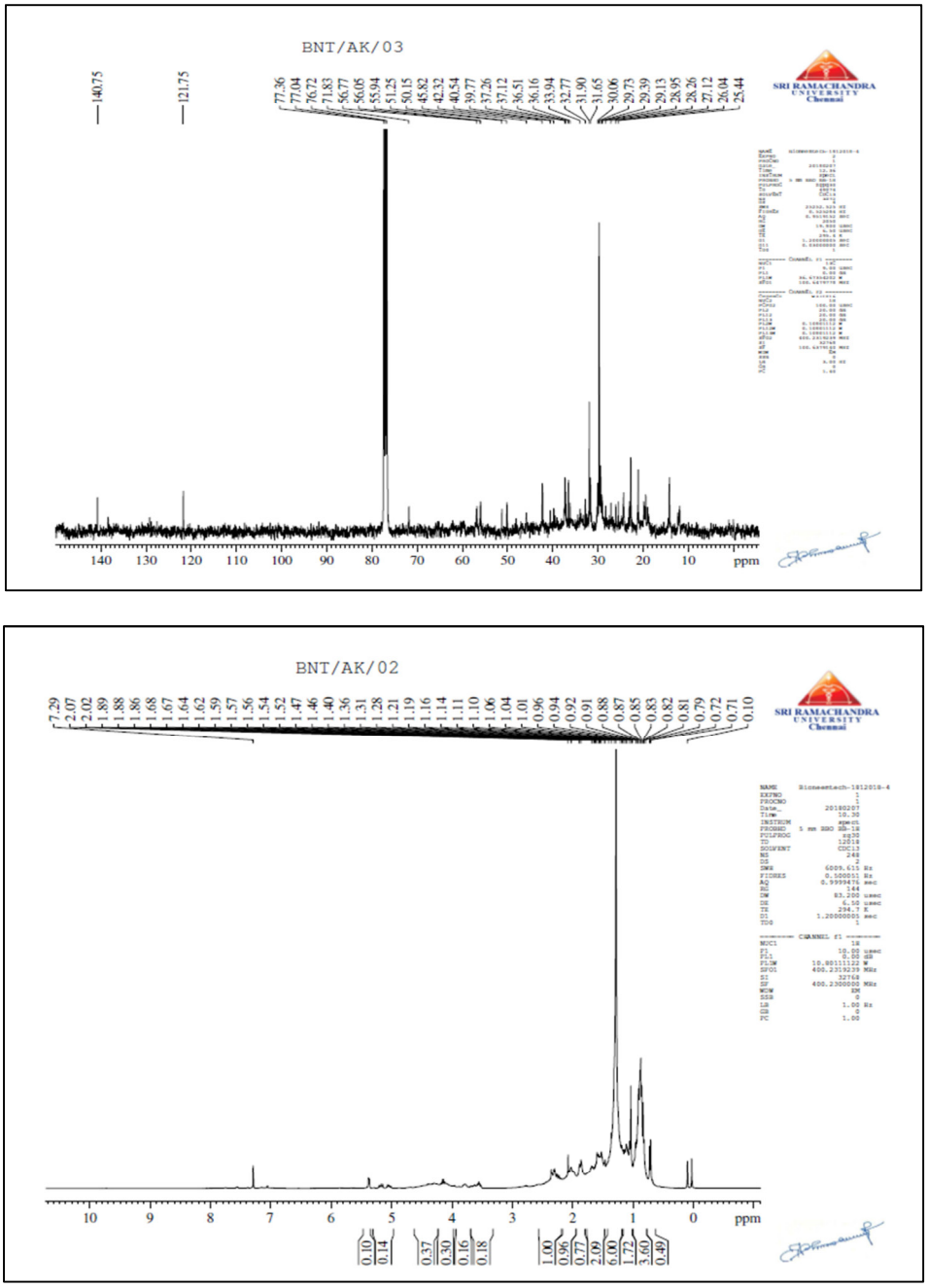
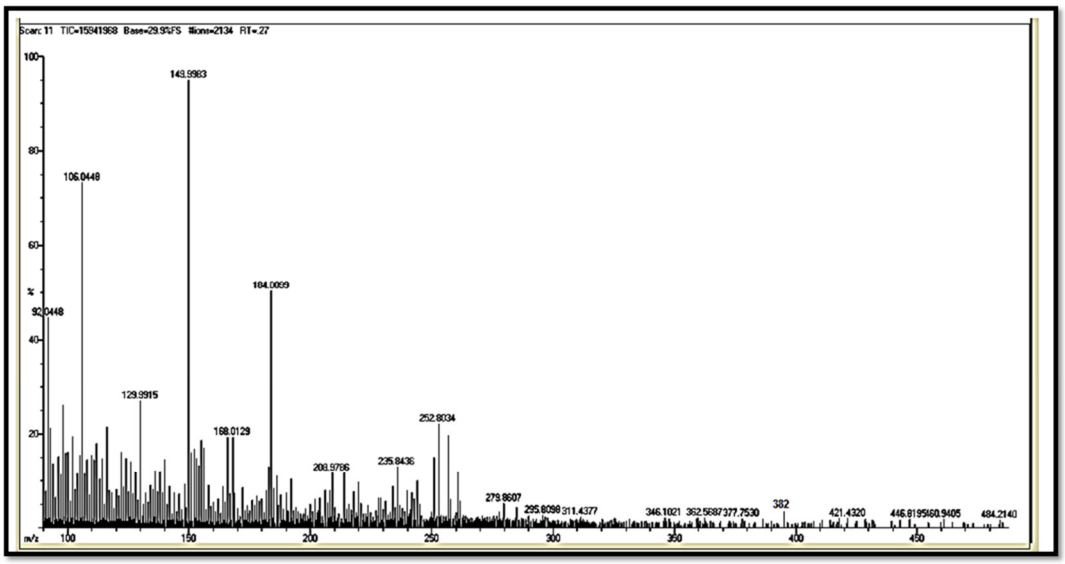


Fig 5: ^{13}C NMR of Compound 2 (A), ^{1}H NMR of compound 2 (B)



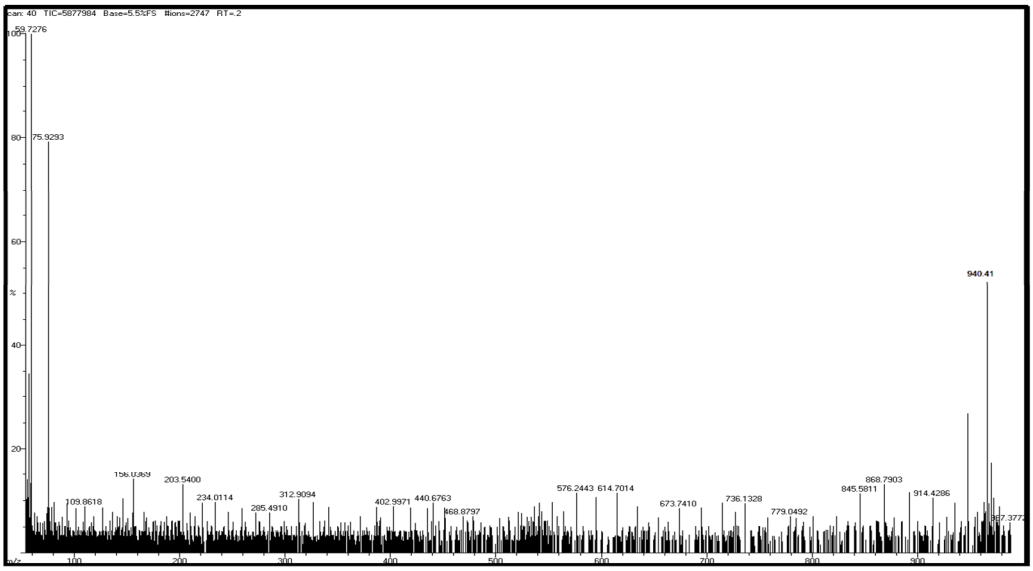


Fig 6: Mass spectra of compound 1(A), and compound 2 (B)

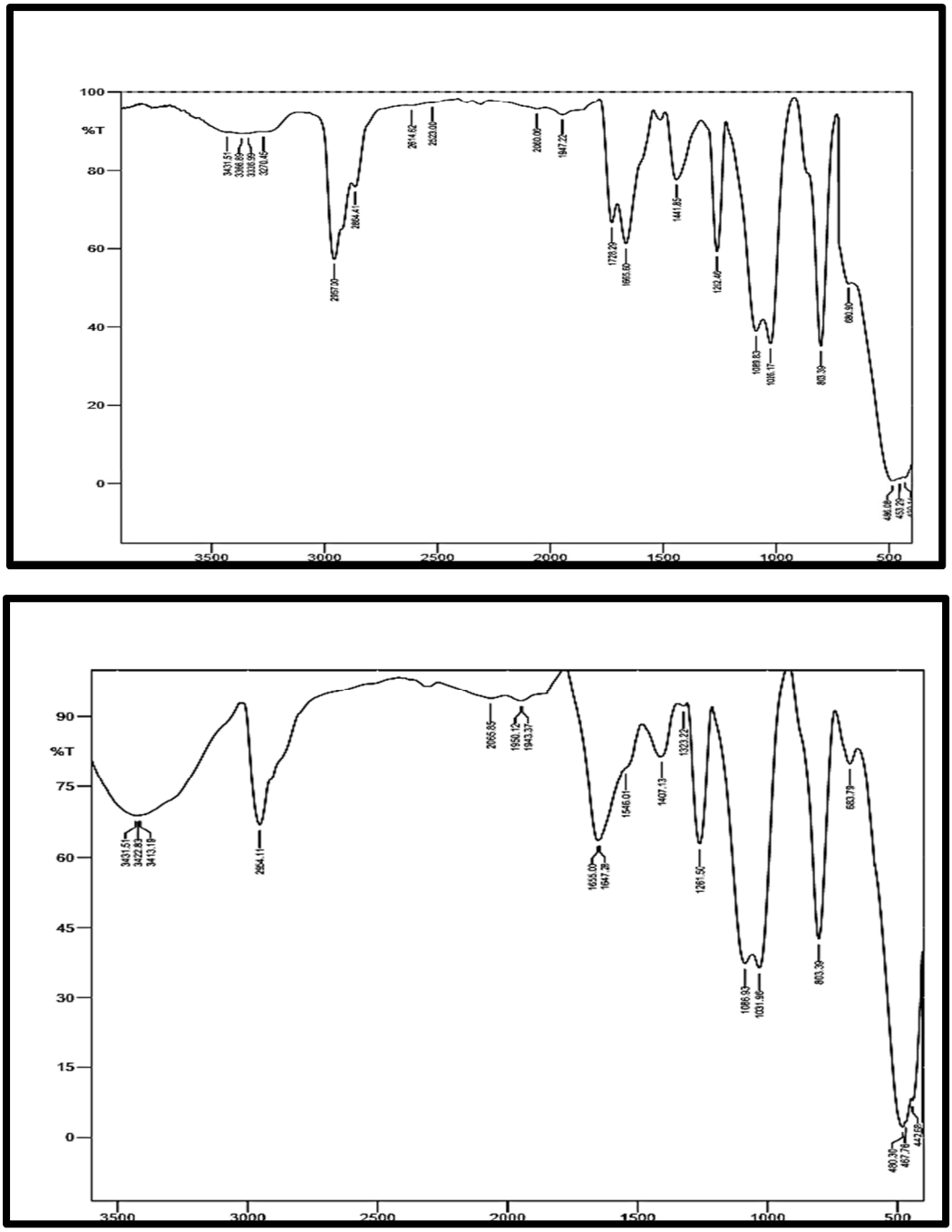


Fig 7: IR spectra of compound 1(a) and compound 2 (b)

4 CONCLUSION

It was reported that different species of the genus "*Crotalaria*" possess diverse biological activities. Thus, the plant *Crotalaria biflora* was selected for our study. Initially, it was decided to do preliminary phytochemical evaluation and characterization of chemical constituents in the extracts of the selected plant *Crotalariabiflora* which was completed successfully and were able to identify the presence of different types of phytochemicals such as alkaloids, terpenoids, sterols, flavones and flavonoids. These phytochemicals are well known for their biological activities. Our further research will be focused on detailed pharmacological screening of the extracts of this plant may give significant outcomes.

5 AUTHORS CONTRIBUTION STATEMENT

K. I. Anoob Kumar carried out the whole experiment with the assistance of Mr V. Sebastin. Dr M. Swamivel Manickam designed the whole research work, Dr M. Sreejith carried out the supervision of experiments.

6 ACKNOWLEDGEMENT

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7 CONFLICTS OF INTEREST

Conflict of interest declared none.

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