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Studies on Phytochemicals and Steroid Isolation from N-Hexane Extract of *Anisochilus carnosus*

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ABSTRACT:

Basic phytochemical screening from the aerial parts of *Anisochilus carnosus* revealed the presence of alkaloids, flavonoids, glycosides, tannins, steroids in high activity, carbohydrates, oils and fats in less activity. The goal of this study is to identify and characterize the bioactive compounds present in this valuable medicinal plant. For the identification and isolation of these phytochemical compounds, different parts of the plant were subjected to extraction of with different solvents namely ethanol, chloroform, acetone and diethyl ether, where ethanolic leaf and flower extracts proved to have high activity. Ethanolic extract of the leaves of this plant showed greater content of these phytochemicals. Further, the dried leaves of the plant are then subjected to extraction with n-hexane and chromatography was performed. The steroid compounds were isolated from the n-hexane extract of the leaves from *Anisochilus carnosus*. Based on the spectral evidence, ^{1}H-NMR and ^{13}C-NMR, structures were determined to be stigmasterol and β-sitosterol.

Keywords: *Anisochilus carnosus, Chromatography, Steroids, Phytochemical Screening, Soxhlet, Stigmasterol and β - sitosterol*

[I] INTRODUCTION

Mankind is blessed with medicinal plants, an expensive gift from nature. In India a number of medicinal plants along with their formulations are being used for various disorders in traditional system of medicine and ethno medical practices. The need for investigations on medicinal plants using indigenous medicinal system has become all the more interesting and relevant [1].

Anisochilus Carnosus (L) Wall is an annual herb. Stem bluntly 4-angled, often tinged with red. Leaves simple, opposite, broadly ovate, obtuse, crenate, base subcordate or rounded, some- what fleshy, usually pubescent beneath, 2.5-6 x 1.2-4 cm. Flowers pale purple, in dense cylindric spikes. Seeds small, suborbicular, compressed, brown. They are commonly found in the Western Ghats distributed in the Southern moist mixed forests, grown among rocks, also known as Karpuravalli and Padukurkka. The soil required for the growth of this plant is sandy loam, slightly acidic soil, which is well drained and medium in organic carbon [2], [3].

Volatile oil of *Anisochilus Carnosus* contain Carvacrol (27.9%), Camphor (14.1%) and α-cis

bergamotene (10.2%) (2-3).The plant has been traditionally used as hepatoprotective agent, stimulant, anti-ulcer, anti- inflammatory [4], [5] and [6]. The leaves of *Anisochilus carnosus* are used traditionally in the treatment of gastric ulcers and stomach ache. The fresh Juice of leaves mixed with sugar candy is given to children in coughs mixed with sugar and gingelly oil and it forms a cooling liniment for the head. The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on human body. The most important of these chemically active constituents of plants are alkaloids, tannin, flavonoid and phenolic compounds. Many of these indigenous, medicinal plants are also used for medicinal purposes [7] and [8].

As the plant is being used extensively in our country as an herbal medicine, it is necessary to have knowledge of the constituents of the plant of our native species. Previous phytochemical investigations resulted in the isolation of ursolic acid, corosolic acid, 24-hydroxy corosolic acid, maslinic acid and 3b, 7b, 24-trihydroxy-urs-12-en-28-oic acid. In this study,we investigated the phytochemical content followed by the n-hexane extract of its leaves were subjected to chromatographic separation to afford two steroids, including stigmasterol and β-sitosterol. Both compounds were isolated from this species [9].

The purpose of this study is to identify and characterize the bioactive principles from the aerial part of *Anisochilus carnosus*. In this paper, we report the isolation and characterization of known compounds namely stigmasterol and beta-sitosterol from *Anisochilus carnosus*

[II] MATERIALS AND METHODS

2.1. Collection of Plant

The fresh plant was collected from Tirupathi and authenticated by Dr. Madhavachetty, Sri Venkateswara University, Tirupathi 517502. Which also comparing with the spotter (specimen) Rapinat herbarium of St. Joseph College at Trichy in Tamil Nadu. **[Specimen No: RHT 563, 10567].**

2.2. Preparation of Organic solvents based extracts

The whole plant materials were dissected into leaves, stem, root, and flowers. After dissection, the plants parts were washed thoroughly in the running tap water, rinsed with distilled water. The completely air dried dissected plant materials viz., leaves, stem and flowers were prepared as extracts using ethanol, chloroform, acetone & diethyl ether appropriately. The different extracts were prepared using soxhlet method and incubated for 7 hours. The ethanol based leaf extract, showed the Very high activity. So we decided to go ahead with the concentrated extract of ethanol based for the further phytochemical based studies [10].

Chem	Ethanol			Chloroform			Acetone			Diethyl ether
Plant parts	Stem	Leaves	Flower	Stem	Leaves	Flower	Stem/ leaves	Flower	Stem/ leaves	Flower
	-	+++ ++	++ ++	-- -	+ +	+	+	+	-	+ +

Table: 1. Activity check in the plant parts treated with different organic solvents

- --- Indicates no activity ; • ++ indicates less activity
- +++++ very high activity ; • + very less activity
- ++++ medium activity

Healthy leaves of *Anisochilus carnosus* were collected from the tissue cultured saplings invitro. The collected leaves were screened for contamination and thoroughly washed. The specimens were then shade dried, ground in a mechanical mixer-grinder, and extracted with ethanol by maintaining the powder: solvent ratio as 1:6 using a Soxhlet apparatus. The crude extract

obtained was concentrated at 40ºC under reduced pressure (72 mbar) with a Rotavapor. The dried extract was weighed to determine the yield of soluble constituents and stored in a vacuum desiccator at room temperature until further use.

Fig: 1. Extraction of dried leaves and Flower using soxhlet apparatus

2.3. Phytochemical screening

Semi-quantitative phytochemical screening of the extract was performed. The extract was analysed for the presence of flavonoids, saponins, terpenoids, reducing sugars, cardiac glycosides, steroids, tannins, phlobatannins, anthraquinones and oil individuals and the results are tabulated.

Phytochemical Analysis

Phytochemical screening was done for analyzing secondary metabolites that are responsible for curing ailments. The phytochemical screening of the plant extract was carried out in all different region collected leaves extracts [11], [12] and [13].

I. **Test for Alkaloids**

Dragendorff's test: To the 1 ml of extract, add 1 ml of Dragendorff's reagent (potassium bismuth iodide solution). An orange-red precipitate indicates the presence of alkaloids.

Mayer's test: To the 1 ml of extract, add 1 ml of Mayer's reagent (Potassium mercuric iodide solution). Whitish yellow or cream coloured precipitate indicates the presence of alkaloids.

Hager's test: To 1 ml of extract add 3ml of Hager's reagent (saturated aqueous solution of picric acid) yellow colored precipitate indicates the presence of alkaloids.

Wagner's test: To the 1 ml of extract add 2 ml of Wagner's reagent (iodine in potassium iodide) formation of reddish brown precipitate indicates the presence of alkaloids.

II. **Test for Saponins**

Take small quantity of alcoholic and aqueous extract separately and add 20 ml of distilled water and shake in a graduated cylinder for 15 minutes lengthwise. Layer of foam indicates the presence of Saponins.

III. **Test for Glycosides**

Legal's test: Dissolve the extract in pyridine and add sodium nitropruside solution to make it alkaline. No formation of pink to red colour shows absence of glycosides.

Baljet's test: To 1ml of the test extract, add 1ml of sodium picrate solution and the yellow to orange color reveals the presence of glycosides.

Keller-Killani test: 1gm of powdered drug is extracted with 10ml of 70% alcohol for 2 minutes, filtered, add to the filtrate, 10ml of water and 0.5ml of strong solution of lead acetate and filtered and the filtrate is shaken with 5ml of chloroform. The chloroform layer was separated in a porcelain dish and removes the solvent by gentle evaporation. Dissolve the cooled residue in 3ml of glacial acetic acid containing 2 drops of 5% ferric chloride solution . Carefully transfer this solution to the surface of 2ml of concentrated sulphuric acid. A reddish brown layer forms at the junction of the two liquids and the upper layer slowly becomes bluish green, darkening with standing.

Borntrager's test: Add a few ml of dilute sulphuric acid to 1ml of the extract solution. Boil, filter and extract the filtrate with chloroform. The chloroform layer is treated with 1ml of ammonia. The formation of red color of the ammonical layer shows the presence of anthraquinone glycosides.

IV. **Test for Carbohydrates**[14],

Molisch's test: To 2ml of the extract, add 1ml of alpha-napthol solution, add concentrated sulphuric

acid through the side of the test tube. Purple or reddish violet color at the junction of the two liquids reveals the presence.

Fehling's test: To 1ml of the extract, add equal quantities of Fehling solution A and B, upon heating formation of a brick red precipitate indicates the presence of sugars.

Benedict's test: To 5ml of Benedict's reagent, add 1ml of extract solution and boil for 2 minutes and cool. Formation of red precipitate shows the presence of sugars.

V. **Test for Flavonoids [15]**

Shinoda's Test:

i) The extract is treated with magnesium foil and concentrated HCl give intense cherry red color indicates the presence of flavonones or orange red color indicates the presence of flavonols.

ii) The extract is treated with sodium hydroxide; formation of yellow color indicates the presence of flavones.

iii) The extract is treated with concentrated H_2SO_4, formation of yellow or orange color indicates flavones.

VI. **Test for Steroids**

Salkowski test: Dissolve the extract in chloroform and add equal volume of conc. H_2SO_4. Formation of bluish red to cherry color in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

Liebermannburchard reaction: A few crystals were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by addition of 2-3 drops of acetic anhydride. Solution turned violet blue and finally green.

VII**. Test for Tannins [16]**

i)Take the little quantity of test solution and mixed with basic lead acetate solution.

Formation of white precipitates indicates the presence of tannins.

ii) To 1ml of the extract, add ferric chloride solution, formation of a dark blue or greenish black color product shows the presence of tannins. The little quantity of the extract is treated with potassium ferric cyanide and ammonia solution. A deep red color indicates the presence of tannins.

iii) To the test extract, add strong potassium dichromate solution, a yellow color precipitate indicates the presence of tannins and Phenolic compounds.

VII. **Test for Fats & Oils**

i) Place a thick section of drug on glass slide. Add a drop of Sudan Red III reagent. After 2 min. wash with 50% alcohol. Mount in glycerin. Observe under microscope. Oil globules appear red.

ii) Place little amount of drug sample on the filter paper and stand for 15 minutes. A greasy spot observe due to presence of fats.

2.4. Isolation of steroids by n- hexane extraction

Extraction and isolation:

The powdered leaves of *Anisochilus carnosus* weighing 1.0 Kg were soaked in 3 litre of ethanol and then 2 litre ethanol for 7 days. The whole mixture was then filtered through filter paper and the filtrate was then evaporated under reduced pressure at (40-50)^oC using a Buchii Rotary Evaporator to provide 130 gm of a gummy concentrate of the crude extract. A portion of the ethanol extract (10.5 gm) was dissolved in 90% ethanol. It was partitioned with n-hexane, then with chloroform ($CHCl_3$) and finally with ethyl acetate (EA). All the extracts were filtered through a cotton plug followed by Whatman filter paper number 1 and then concentrated by using a rotary vacuum evaporator to provide n-hexane (2.5 gm), then with chloroform ($CHCl_3$) (2.0 gm) and finally with ethyl acetate (1.0 gm) extractives [17].

Chromatographic separation:

The column was packed with fine TLC grade silica gel (Himedia 60 – 120 mesh) was used as the packing material. A column having 40 cm length and 3 cm in diameter was packed with the silica gel (70 gm) up to a height of 23 cm under reduced pressure. The column was washed with n-hexane to facilitate compact packing. The sample was prepared by adsorbing 3.5 gm of n-hexane soluble extract onto silica gel (Himedia 60 – 120 mesh), allowed to dry and subsequently applied on

top of the adsorbent layer. The column was then eluted with n-hexane followed by mixtures of n-hexane and dichloromethane and then dichloromethane and methanol [18].The polarity was gradually increased by adding increasing proportions of dichloromethane and methanol. A total of 30 fractions were collected each in 100 ml beakers.

The Fractions of the crude n-hexane extract was subjected to Sub-Column Chromatography) (Himedia 60 – 120 mesh) for further fractionation. The column was eluted with n-hexane, ethyl acetate and methanol mixtures of increasing polarities to provide 160 fractions. Similar fractions were pooled together. Further purification is carried out using preparative TLC. Spots were identified, scraped and eluates using petroleum ether and chloroform as solvents The observation for the crystal formation is to be found and TLC will further be performed.

Finally eluate ST yielded a single spot when subjected to TLC using several solvent systems including chloroform: ethanol (9.8:0.2), ethyl acetate: ethanol (9.8: 0.2), chloroform: ethyl acetate (4:1) and it showed to be homogenous compound. ST a white crystalline powder (100mg) with melting point (144-146°C) was further subjected to Proton NMR (400MHz).

The crystals were subjected to confirmatory test such as test for alcohol and steroid.

Tests for alcohol

4g of cerric ammonium nitrate was dissolved in 10ml of 2N HNO3, on mild heating. A few crystals of isolated compound were dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of ceric ammoinium nitrate reagent and diluted to 1ml with dioxane and shaken well. The developed yellow to red color indicates the presence of an alcoholic hydroxyl group [11].

Tests for steroid

Salkowski test: Dissolve the extract in chloroform and add equal volume of conc. H_2SO_4. Formation of bluish red to cherry color in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

Liebermannburchard reaction: A few crystals were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by addition of 2-3 drops of acetic anhydride. Solution turned violet blue and finally green.

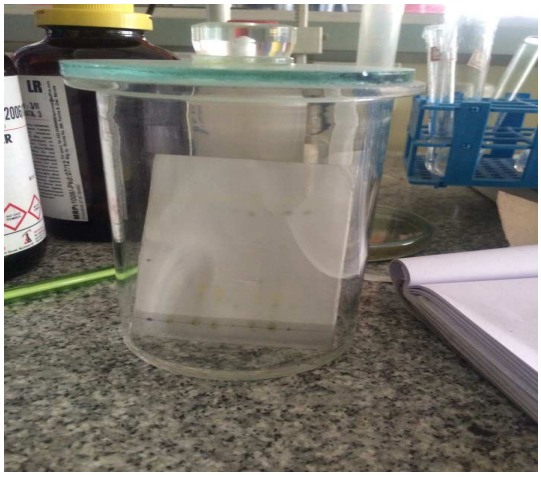


Fig: 2. TLC Chamber

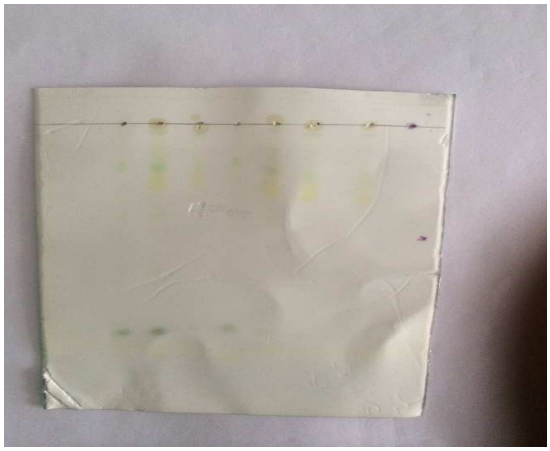


Fig: 3. [a]After running the Mobile phase

Fig: 3. [b] Iodine Chamber Treatment

[III] RESULTS

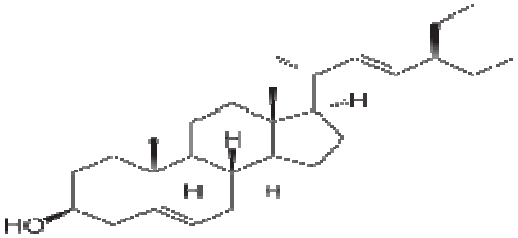
In the present investigation, preliminary Phytochemical screening has been done in the ethanol based leaf of *Anisochilus carnosus plant parts* showed the presence of phytochemical constituents namely alkaloids, carbo- hydrates, flavonoids, glycosides, saponins, steroids, tannins, phenols, triterpenoids, and the absence of anthraquinones, oils, fats and amino acids.

S.No	Phytochemical screening tests	Ethanol based leaves extract	Ethanol based flower extract
1	Alkaloids	Very high activity	Very high activity
2	Amino acids	No amino acids	No amino acids
3	Anthraquinones	Very high activity	Very high activity
4	Flavonoids	Very high activity	Very high activity
5	Glycosides	Very high activity	Very high activity
6	Saponins	Very high activity	Very high activity
7	Steroids	Very high activity	Very high activity
8	Tannins	Very high activity	Very high activity
9	Triterpenoids	Very high activity	Very high activity
10	Oil & resins	No activity	No activity
11	Carbohydrates	Very less activity	Very less activity

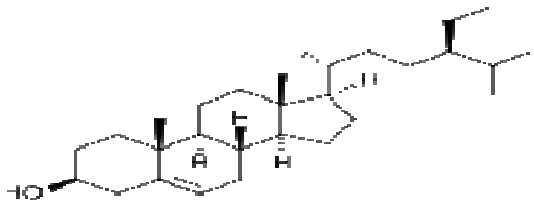
Table: 2. Phytochemical Studies of Leaves and Flower using ethanol extract

Spectroscopic Characterization for steroids:

The IR and C- NMR have been carried out to elucidate the structure of the isolated compounds.



Stigmasterol ($C_{29}H_{48}O$; Mol.Wt. 412.69)



βsitosterol($C_{29}H_{50}O$; Mol.Wt: 414.71)

^{1}H-NMR and ^{13}C-NMR spectra were recorded using CDCl3 as solvent on

AV400 - 400 MHz High Resolution Multinuclear FT-NMR spectrometer., IISC, Bangalore.

Stigmasterol:

- ^{1}HNMR (400MHz, CDC_{l3}) δ: 5.34 (1H, d, J 5.2 Hz, H6), 5.16 (1H, dd, J 15.0, 8.4 Hz, H22), 5.03 (1H, dd, J 15.0, 8.4 Hz, H23), 3.52 (1H, dd, J 9.6, 4.8 Hz, H3), 1.00, 0.67 (3H, s, H19 and H18), 0.92 (3H, d, J 6.0 Hz, H21), 0.85 (3H, d, J 8.0 Hz, H29), 0.81 (3H, d, J 7.2 Hz, H26) and 0.79 (3H, d, J 7.2 Hz, H27).
- ^{13}CNMR (100 MHz, CDC_{l3}) δ: 37.29 (CH2,C1), 28.28 (CH2,C2), 71.85 (CH,C3), 42.36 (CH2,C4), 140.81 (Cq,C5), 121.74 (CH,C6), 31.72 (CH2,C7), 34.01 (CH,C8), 50.2 (CH,C9), 36.54 (Cq,C10), 26.17 (CH2,C11), 39.83 (CH2,C12), 42.37 (Cq,C13), 56.82 (CH,C14), 24.33 (CH2,C15), 29.23 (CH2,C16), 56.12 (CH,C17), 12.02 (CH3,C18), 19.41 (CH3,C19), 40.50 (CH2,C20), 21.13 (CH3, C21), 138.33 (CH,C22), 129.0 (CH,C23), 51.28 (CH,C24), 45.91 (CH,C25), 19.42 (CH3, C26), 19.84 (CH3,C27), 24.34 (CH2,C28), 12.26 (CH3,C29).

β-Sitosterol:

- ^{1}HNMR (400 MHz,CDC_{l3}) δ: 5.34 (1H, d, J 5.2 Hz, H6), 3.51 (1H, m, H3), 1.00, 0.67 (3H, s, H19 and H18), 0.92 (3H, d, J 6.0 Hz, H21), 0.85 (3H, d, J 8.0 Hz, H29), 0.83 (3H, d, J 7.2 Hz, H26) and 0.79 (3H, d, J 7.2 Hz, H27).
- ^{13}CNMR (100 MHz, CDC_{l3}) δ: 37.29 (CH2,C1), 31.95 (CH2,C2), 71.84 (CH,C3), 42.36 (CH2,C4), 140.80 (Cq,C5), 121.73 (CH,C6), 31.71 (CH2,C7), 31.95 (CH,C8), 50.19 (CH,C9), 36.18 (Cq,C10), 21.12 (CH2,C11), 39.82 (CH2,C12),

42.36 (Cq,C13), 56.81 (CH,C14), 24.33 (CH2,C15), 28.26 (CH2,C16), 56.11 (CH,C17), 11.88 (CH3,C18), 19.41 (CH3,C19), 36.54 (CH,C20), 19.07 (CH3,C21), 34.00 (CH2,C22), 26.16 (CH2,C23), 45.89 (CH,C24), 29.23 (CH,C25), 19.83 (CH3,C26), 18.81 (CH3,C27), 23.12 (CH2,C28), 12.01 (CH3,C29).

From the above findings, β-sitosterol and stigmasterol were isolated from hexane extract of the leaves of *Anisochilus carnosus* and chemical structures obtained respectively. It was carried out by means of various physical (solvent extraction, TLC, Column chromatography) and NMR spectral techniques.

[IV] DISCUSSION

Medicinal plants were of great importance to the health of individuals and the community [19] , [20]. Phytochemical analysis conducted on the plant extract showed the presence of constituents which are known to exhibit medicinal as well as physiological activities [21]. Analysis of the plant extracts revealed the presence of phytochemicals, such as proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids.

In the present study the different parts of the *Anisochilus carnosus* plant was selected and subjected to various solvents using soxhlet apparatus. It indicated that ethanolic leaf extracts showed high activity when compared to other parts of the plant extracts which showed moderate activity. Table (1) shows the best solvent used for extraction from the different parts of *Anisochilus carnosus*. The preliminary investigation of phytochemical studies was reported from the leaves of A. carnosus (L) Wall.

It exhibited positive to Foam test and Haemolytic test for saponins, Shinoda test for flavonoids and Salkowaski and Lieberman-Buchard reactions for steroids. These results were well in accordance with the earlier antidiabetic potential of several plants [19].

Compound 1 was isolated as a white needle shaped crystal with melting point 138-140^{o}C which gave positive Salkowski and Lieberman-Burchard test for steroid. The mass spectral data of the compound gave a molecular formula $C_{29}H_{48}O$.The ^{1}HNMR spectrum showed two one proton multiplets at δ 3.52 and δ 5.34 typical for H3 and H6 of a steroidal nucleus. Two olefinic protons appeared as characteristic downfield signals at δ 5.16 (1H, dd, J = 15.0, 8.4 Hz) and 5.03 (1H, dd, J = 15.0, 8.4 Hz) in the 1H NMR spectrum which were identical with the chemical shift of H22 and H23 respectively of stigmasterol [22,23]. The spectrum also displayed two three proton singlets at δ 1.00 and δ 0.67 assignable for H19 and H18 respectively. In addition, two doublets at δ 0.92 (3H, d, 6.0 Hz) and 0.85 (3H, d, 8 Hz) could be ascribed to the two methyl groups at H29 and H21and another three-proton doublet at δ 0.81 (3H, d, 7.2 Hz) for H26. On the other hand, one three-proton triplet at δ0.79 (3H, J 7.2 Hz) could be assigned to the primary methyl group attached H27 [24].

The ^{13}C NMR spectrum showed 29 carbons which included an oxymethine carbon at δ 71·85, was characteristics of spirostene [25] and two olefinic carbons appeared at δ 138.33 and 129.0 which were identical with the chemical shift of C22 and C23 respectively of stigmasterol. If we compare DEPT 90 and 135 experiments for 1 then we confirmed that this compound was having six methyl (CH3) groups, nine methylene (CH2), eleven methine (CH) and three quaternary carbons (Cq) groups. The physical and spectral data of the compound was in complete agreement to the reported data in literature value [25], [26], [27] and [28]. The compound 1 was identified as stigmasterol.

Compound 2 was obtained as white crystalline compound with melting point 135-137^{o}C which gave positive confirmatory tests. Compound 2 was identified as β-sitosterol by comparison the 1H and ^{13}C NMR spectra with that of 1. The NMR data were very similar to those of the compound 1 except two olefinic protons were absent while two methylene signals were present for H22 and H23 and showed two one-proton multiplet at δ 3.51

ppm and δ 5.34 ppm typical for H3 and H6 of a steroidal nucleus. The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbonsignal at δ 71·84 and two olefinic carbons at δ 140·80 and δ 121·73. The double bonded unsaturation at δ 140.80 and δ 121.73 was characteristics of spirostene [26] and two olefinic carbons were absent at 138.33 and 129.0 while two methylene carbon signals were present at 34.0 and 26.16 for C22 and C23. The comparison between DEPT 135 & DEPT 90 experiments for 2 was done and it proved that this compound was having six methyl (CH3) groups, eleven methylene (CH2) groups, nine methine (CH) groups and three quaternary carbons (C) groups. These assignments are in good agreement for the structure of β-sitosterol [22], [24] , [29], [30], and [31].

[V] CONCLUSION

From the above findings, the phytochemical screenings were performed and the presence of alkaloids, Flavanoids, Steroids, Glycosides were found to be present in high amounts in ethanol mediated leaf extract of the Plant. Stigmasterol and β – sitosterol were isolated from n – hexane extract of the leaves of A. carnosus and the chemical structures were identified respectively. It was carried out by various means such as (TLC, Column chromatography and NMR).

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