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In Vitro Anti Oxidant Activity of Extracts and Stigmasterol from Leaves of *Clerodendrum inerme* Linn

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ABSTRACT

Stigmasterol is an important constituent from plant, as it is involved in the synthesis of many hormones like progesterone, estrogens, androgens and corticoids. In the present study Stigmasterol was isolated by column chromatography from chloroform leaf extract of *Clerodendrum inerme*. The isolated stigmasterol was identified by melting point and characterized by IR, ^{1}H NMR, ^{13}C NMR and GCMS studies. The extracts and stigmasterol was investigated for *in vitro* antioxidant activity for three methods such as DPPH radical, Superoxide radical and Hydroxyl radical scavenging assay. Ethanol extract shows good *in vitro* antioxidant activity than chloroform extract. Stigmasterol shows significant *in vitro* antioxidant activity than the extracts.

Keywords: Column chromatography, Stigmasterol, *in vitro* antioxidant, DPPH**,** Superoxide dismutase

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INTRODUCTION

Clerodendrum inerme (L.) Gaertn. (Family: Verbenaceae), is a common shrub that grows in India, both in the wild and as a garden hedge. Its leaves are used as alterative, febrifuge and as a substitute for *Swertia chirayita.* Its leaves have been shown to possess antimicrobial activity [1] and are reported to be cardiovascular system active. They also stimulate uterine motility in rats and inhibit intestinal motility [2]. In Indian tribal medicine, leaves of *Clerodendrum inerme* are used for treating fever, cough, skin rashes, chronic pyrexia and boils, and are used in conjunction with other plant leaves. They are also used to treat umbilical cord infection and for cleaning the uterus in local medicine [3,4]. Aerial parts of *Clerodendrum inerme* showed potent anti-viral activity against Hepatitis B virus [5]. Whole plant parts of *Clerodendrum inerme* are used as to treat coughs, scrofulous infection, venereal infection, skin diseases and Beriberi diseases [6]. It is also used as febrifuge, vermifuge and antioxidant. The plant contains mainly iridoids, flavonoids, sterols and triterpenes.

Stigmasterol has been investigated for its Pharmacological prospects such as cytotoxicity, antihyperlipidemic, anti osteoathritic, antitumour, antimutagennic, anti inflammatory and CNS effects. The aim of the present investigation was to evaluate *in-vitro* antioxidant activity of the extracts and isolated compound stigmasterol in three methods such as DPPH radical, Superoxide radical and Hydroxyl radical scavenging assay.

MATERIALS AND METHODS

Plant Material

The plant material used in this study consists of leaves of *Clerodendrum inerme* collected from herbal garden, Siddha Medical College & Research Institute, Chennai district, India during spring March - April 2010. It was taxonomically authenticated by Dr. Sasikala Ethirajulu, Botanist, Siddha Medical College & Research Institute, Chennai. The voucher specimen was deposited in the herbarium of College Of Pharmacy, Madras Medical College, Chennai-03, India. Exposure to sunlight was avoided to prevent the loss of active components. After drying the leaves of *Clerodendrum inerme* were dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve.

Extraction:

The air dried powdered leaves (500g) were subjected to soxhlet extraction using chloroform and ethanol solvents successively. Evaporation of the solvents at normal temperature yielded 30% w/w residue. The dried leaf powder was packed in Soxhlet apparatus and successively extracted with chloroform by for 24 hrs and then the mark was subjected to ethanol for 24 hrs. The extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained. [7]

Column Chromatographic Separation

In order to carry out column chromatography, a solvent system was established by developing TLC technique. The silica gel (60 – 120 mesh size) slurry was made with the solvent system established earlier. The slurry was poured time to time into column very carefully and the silica gel was allowed to settle down to form uniform packing. Then the stop – cock of the column was opened and the excess of solvent over the column head was allowed to run. The dried crude chloroform extract of *Clerodendrum inerme* was mixed with small amount of silica gel in a mortar to get a free flowing powder. The powdered sample was then applied carefully on top of the prepared column and successfully eluted with solvent system. The column was run by hexane, chloroform and methanol by gradient elution technique.TLC was used to monitor the eluates. Total of 50 eluates were collected. Similar fractions were pooled together. Further purification is carried out using preparative TLC. Spots were identified, scraped and eluted using pet ether and chloroform as solvents to yield white crystalline powder, stigmasterol. (ST).

Thin layer chromatography

The eluate ST yielded a single spot when subjected to TLC using various solvent systems such as chloroform: ethanol (9.5: 0.5), ethyl acetate: ethanol (9:1), chloroform ethyl acetate (9.5: 0.5) and it showed to be a homogenous compound. ST a white crystalline powder (100mg) with a melting point (144 – 146ºC) and further subjected to IR, ^{1}H-NMR and ^{13}C- NMR(400MHz) and GC MS to ascertain the chemical structure. [8, 9]

Test for alcohols

4g of cerric ammonium nitrate was dissolved in 10ml of 2N HNO_3, On mild heating, few crystals of isolated compound were dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of cerric ammonium nitrate reagent and diluted to 1ml with dioxane and shaken well. The developed yellow to red colour indicates the presence of alcoholic hydroxyl group.

Test for steroid

Salkowski reaction

A few crystals were dissolved in chloroform and a few drops of concentrated sulphuric acid were added to the solution. A reddish color was seen in the upper chloroform layer. [10]

Liebermann Burchard reaction

A few crystals were dissolved in chloroform and a few drops of concentrated sulphuric acid were added to it followed by the addition of 2 – 3 drops of acetic anhydride. Solution turned violet blue and finally green. [10]

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Spectroscopical characterization

Melting point was determined by determined in open –glass capillaries on Stuart SMP10 melting point apparatus and were uncorrected. The IR (KBr) spectrum was recorded on a Shimadzu UV 168A and Perkin Elmer 1600 FTIR spectrometer, respectively. The 1H-NMR and 13C-NMR spectra were recorded on a Bruker R32 (400 MHz) in DMSO –d with TMS as an internal standard (chemical shifts in δ, ppm). TLC was performed with silica gel 60 G F254 and spots were visualized by iodine vapors or ultraviolet light. GC-MS were recorded at high resolution on mass spectrometer (Perkin Elmer Auto system) and data was given in m/z values.

The IR absorption spectrum showed absorption peaks at 3373.6 cm-1 (O-H stretching); 2940.7 cm^{-1} and 2867.9cm^{-1} (aliphatic C-H stretching); 1641.6cm^{-1}(C=C absorption peak); other absorption peaks includes 1457.3cm^{-1} (CH_2); 1381.6cm^{-1} (OH def), 1038.7cm^{-1}(cyclalkane) and 881.6cm^{-1}

^{1}H NMR (CDCl3, 400 MHz) of Stigmasterol

1H NMR has given signals at δ3.2 (1H, m, H- 3), δ5.26 (1H, m, H -6), δ 5.19(1H, m, H – 23), 4.68(1H, m, H-22), 3.638(1H, m, H-3), 2.38(1H, m, H -20), δ1.8 – 2.0 (5H, m) ppm. Other peaks also observed at δ0.76 – 0.89 (m, 9H), 0.91 – 1.05(m, 5H), 1.35- 1.42 (m, 4H)

^{13}CNMR (CDCl3, 400 MHz) of Stigmasterol

^{13}CNMR has given signal at 150.98, 145.2(C-5), 139.8 (C- 22), 121.7, 118.89 (C-6), 79.03 (C-3), 55.3 (C- 14), 55.18 (C- 17), 50.45 (C- 9), 48.3 (C- 9), 40.8 (C- 20), 40.1 (C- 12), 39.2(C- 13), 38.9 (C-4), 38.6 (C- 12), 37.18 C- 1), 37.12 (C- 10), 36.3 (C- 8),35.59 (C- 20), 34.29(C- 22), 34.24(C-7). [11]

FAB – MS spectroscopy showed the molecular ion peak at 412 that correspond to the molecular formula, $C_{29}H_{48}O$. Ion peaks were also observed at m/z 367, 271, 255, 229, 189, 175, 161, 133, 121, 105,107, 95, 81,69,55,41.

***In Vitro* Antioxidant Activity**

DPPH- Radical Scavenging Method

Methanolic solution of 0.5ml of DPPH (0.4mM) was added to 1 ml of the different concentrations of plant extract/compounds and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol without the compounds served as the positive control. After 30 min, the absorbance was measured at 518 nm and converted into percentage radical scavenging activity as follows. Quercitin and Rutin manufactured from Celestial Biolabs Limited, Hyderabad, India.

$$Scavenging activity(\%) = \frac{A_{518}\ \text{Control} - A_{518}\ \text{Sample}}{A_{518}\ \text{Control}} \times 100$$

Where A_{518} control is the absorbance of DPPH radical+ methanol; A_{518} sample is the absorbance of DPPH radical or compound / standard. DPPH was from Sigma Aldrich, Mumbai, India. [12]

Super oxide anion radical scavenging activity

Superoxide radical (O_2^-) was generated from the photoreduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT) reduction method (Winterbourne et al., 1975). Measurement of superoxide anion scavenging activity was performed The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Quercetin was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples. Superoxide radical was manufactured from Sigma Aldrich, Mumbai, India. [13]

Hydroxyl radical scavenging activity

The assay is based on quantification of degradation product of 2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} - Ascorbate –EDTA –H_2O_2 system (Fenton reaction). The reaction mixture contained 0.1 ml deoxyribose (2.8mM),0.1 ml EDTA (0.1 mM), 0.1 ml H_2O_2 (1mM), 0.1 ml Ascorbate (0.1mM), 0.1ml KH_2PO_4-KOH buffer, pH 7.4 (20mM) and various concentrations of plant extract in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37^0C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated. [14]

RESULTS AND DISCUSSION

From the positive test for steroids and alcohols given by, ST it is assumed to be compound containing steroid nucleus. The ST is a white crystalline compound containing steroid nucleus. The ST is white crystalline needles like substance with melting point 144-146ºC, on subjection to IR spectroscopic analysis, the observed absorption bands are 3373.6cm-1 that is characteristic of O-H stretching. Absorption at 2940.7 cm^{-1} and 2867.9cm^{-1} is due to aliphatic CH – stretching. Other absorption frequencies include 1641.6cm^{-1} as a result C=C stretching however this band is weak at 1457.3cm^{-1} is a bending frequency for cyclic $(CH_2)n$ and 1381.6cm^{-1} for –CH_2 $(CH_3)_2$ γ. The absorption frequency at 1038cm^{-1}signifies cycloalkane. The out of plane C-H vibration of unsaturation part observed at 8812 cm^{-1}. These absorbtion frequencies resemble that of stigmasterol. The ^{1}H NMR showed the proton of H- 3 appeared multiplet at δ3.2 and also reveals the existence of signals for olefenic proton at δ5.19 multiplet, 4.68(m), 4.6(m)and 2.38(m). The angular methyl proton at δ0.69 singlet, δ0.80 singlet, δ1.02 singlet corresponds to C -18 and C -19 protons respectively.

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The ^{13}C NMR has shown respective signals at 145.2 and 125.7ppm, which are assigned to $C_5 – C_6$ double bonds respectively as in Δ^5 spirostene. The angular carbon atom assigned at 19.32. The alkene carbon resonates at δ145.2, 139.8, 121.7and 118.89. The spectra shows twenty nine carbon signal including six methyl, nine methylenes, eleven methane and three quaternary carbons.

FAB – MS spectroscopy showed the molecular ion peak at 412 that correspond to the molecular formula, $C_{29}H_{48}O$. Ion peaks were also observed at m/z 367, 271, 255, 229, 189, 175, 161, 133, 121, 105,107, 95,81,69,55,41. Hence from the above spectral characterization the corresponding structure was found to be stigmasterol. (Figure 1)

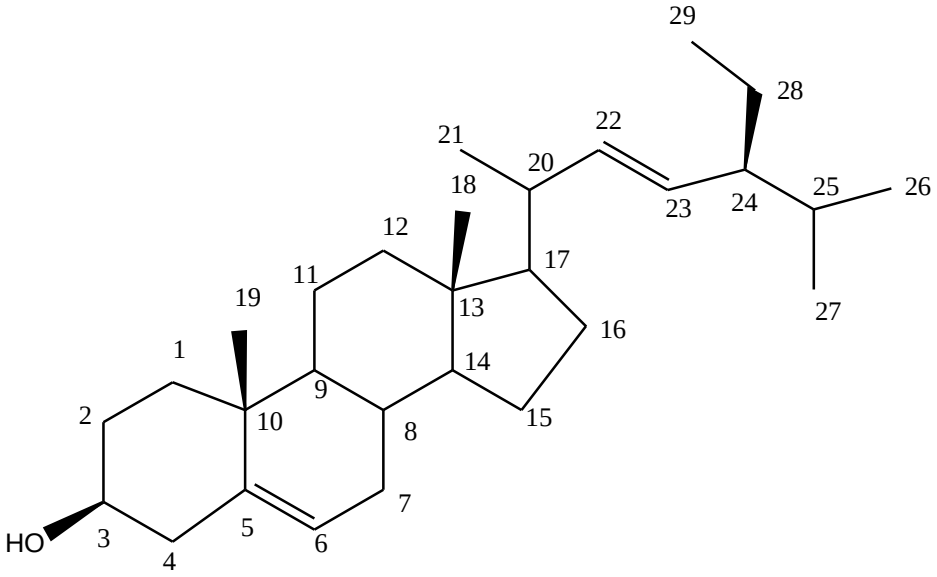

Fig - 1: Stigmasterol ($C_{29}H_{48}O$) Mol Wt. 412.69

The *in vitro* antioxidant activity against DPPH radical indicates that, at 1000µg/ml ethanol extract shows good antioxidant activity than chloroform extract with the percentage scavenging activity of 55.32% and IC_{50} value of 350µg/ml. Stigmasterol shows significant antioxidant activity against DPPH radical than that of extracts with the IC_{50} value of 220µg/ml. Standard quercitin exhibits greater antioxidant activity with the IC_{50} value of 135 µg/ml. (Table 1) The *in vitro* antioxidant activity against superoxide radical indicates that, at 1000µg/ml ethanol extract shows good antioxidant activity than chloroform extract with the percentage scavenging activity of 58.52% and IC_{50} value of 320µg/ml. Stigmasterol shows significant antioxidant activity against DPPH radical than that of extracts with the IC_{50} value of 210µg/ml. Standard quercitin exhibits greater antioxidant activity with the IC_{50} value of 120 µg/ml. (Table 2) The *in vitro* antioxidant activity against superoxide radical indicates that, at 1000µg/ml ethanol extract shows good antioxidant activity than chloroform extract with the percentage scavenging activity of 58.52% and IC_{50} value of 320µg/ml. Stigmasterol shows significant antioxidant activity against DPPH radical than that of extracts with the IC_{50} value of 210µg/ml.

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Standard quercitin exhibits greater antioxidant activity with the IC_{50} value of 120 µg/ml. (Table 3)

Table 1: DPPH radical scavenging activity of the extracts and stigmasterol of *Clerodendrum inerme*

Treatment	Concentration (µg/ml)				IC_{50} values (µg/ml)
	125	250	500	1000	
Chloroform extract	25.27±0.08	30.72±0.32	38.70±0.16	43.17±0.02	550
Ethanol extract	38.42±0.09	48.03±0.11	50.67±0.05	55.52±0.03	350
Stigmasterol	40.32±0.06	50.46±0.21	55.51±0.14	63.69±0.01	220
Quercitin	48.36±0.16	56.07±0.14	62.10±0.11	69.54±0.12	135

All values are expressed as mean ± SEM for three determinations

Table 2: Superoxide radical scavenging activity of the extracts and stigmasterol of *Clerodendrum inerme*

Treatment	Concentration (µg/ml)				IC_{50} values (µg/ml)
	125	250	500	1000	
Chloroform extract	30.25±0.08	32.48±0.32	36.26.±0.1	44.17±0.02	501
Ethanol extract	36.42±0.09	45.03±0.11	53.67±0.05	58.52±0.03	320
Stigmasterol	38.32±0.06	55.46±0.21	57.51±0.14	65.69±0.01	210
Quercitin	50.36±0.16	55.07±0.14	65.10±0.11	69.54±0.12	120

All values are expressed as mean ± SEM for three determinations

Table 3: Hydroxyl radical scavenging activity of the extracts and stigmasterol of *Clerodendrum inerme*

Treatment	Concentration (µg/ml)				IC_{50} values (µg/ml)
	125	250	500	1000	
Chloroform extract	25.25±0.08	26.48±0.32	28.26.±0.1	35.17±0.02	601
Ethanol extract	32.42±0.09	47.03±0.11	55.67±0.05	58.52±0.03	302
Stigmasterol	40.32±0.06	52.46±0.21	63.51±0.14	67.69±0.01	150
Quercitin	47.36±0.16	57.07±0.14	68.10±0.11	70.54±0.12	110

All values are expressed as mean ± SEM for three determinations

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CONCLUSION

Clerodendron inerme is an ornamental plant, the present investigation justifies the traditional usage of their promising antioxidant activity, Ethanol extract shows good antioxidant activity than chloroform extract against the radical used. Stigmasterol was isolated from chloroform extract of *Clerodendron inerme* and found to have potent antioxidant activity than both the extracts.

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