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## Research Article

# QUANTIFICATION OF STIGMASTEROL IN SUCCESSIVE EXTRACTS OF *CLERODENDRUM SERRATUM*, POLY HERBAL FORMULATION BY HPTLC METHOD AND INVITRO ANTI - OXIDANT ACTIVITY STUDIES

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**ABSTRACT**

The aim of the present study is to carry out the quantification of *Clerodendrum serratum* containing poly herbal formulations by HPTLC and to study the *invitro* anti-oxidant activity of nitric oxide scavenging activity of the roots of *Clerodendrum serratum*.The rhizome part of *Clerodendrum serratum* has been selected, dried, powdered and extracted with petroleum ether, chloroform, ethyl acetate and methanol successively by continuous hot percolation method using soxhlet apparatus. Preliminary phytochemical screening of the successive extracts showed the presence of chemical constituents like triterpenoids, flavonoids, alkaloids, tannins, glycosides and sesquiterpenes. The $R_f$ value of stigmasterol was found to be 0.80 ± 0.02.The developed methods were validated as per ICH guidelines. The LOD and LOQ was found to be 100 and 300 ng/spot respectively for HPTLC. The anti-oxidant activity was highest in petroleum ether extract when compared to the other extracts by using ascorbic acid as control. This shows that the polyherbal formulation containing stigmasterol has medicinal value and that it may be utillised for its anti-oxidant properties.

**Keywords:** *Clerodendrum serratum,* stigmasterol, HPTLC, ascorbic acid.

## INTRODUCTION

Standardization of herbal formulations is essential in order to make an assessment of the quality drugs, based on the concentration of their active principles, physical, chemical, phyto-chemical, standardization, In-vitro and In-vivo parameters. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine [1]. The botanical name 'bharngi' refers to *Clerodendrum serratum* and it belongs to family Verbenaceae. The bark mixture contains four major triterpenoid constitutents such as stigmosterol, oleonolic acid, queretaroic acid and serratagenic acid. It grows to 0.3-3.0 m in height and the leaves are 7 to15 cm long. The flowers are blue in colour, and the fruits are purple in colour[2]. The rhizome part of *Clerodendrum serratum* has been selected for the analytical work because it contains high amounts of triterpenes which are responsible for the main pharmacological actions like anti-oxidant, anti-hypertensive, anti-inflammatory actions, etc[3]. Bharngi is bitter in taste and pungent, the pungent taste is used for the post digestive effect. It alleviates tridosas, vata, pita and kapha. It is used for treatment of conditions as asthma, cough, fever, etc[4]. The solvents of increasing polarity selected were petroleum ether, chloroform, ethyl acetate and methanol. The extracts were subjected to preliminary phytochemical screening, HPTLC quantification for the marker and *invitro* antioxidant activity

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studies[5]. Effective formulations are present in the market for plant extracts individually and in combination. In this study, the formulation which was selected for quantitative determination of stigmasterol by validated HPTLC (SUPRES SYRUP) contained 6.273gm the rhizomes of *Clerodendrum serratum*.

**MATERIALS AND METHODS**

**Plant material**

The collected whole Plant was identified and certified by Dr.C.Kunhikannan, Scientist D, Institute Of Forest Genetics Tree Breeding, Coimbatore, TamilNadu. The authentified certificate of the plant *Clerodendrum serratum* is enclosed.

**Process of extraction**

The powder was extracted with various organic solvents viz., petroleum ether, chloroform, ethyl acetate and methanol successively by continuous hot percolation method using soxhlet apparatus. The duration of each extraction was 5 days to get a well extracted product by the respective solvents. After each extraction, the extract was collected & dried either under air at room temperature or by heating the extract at temperature below the boiling point of the extracting solvent to get a well dried extract. Then the dried extract was weighed and the percentage yield of the extract from the weighed rhizome powder was calculated and their percentage yields are shown in the table. The extracts were stored in a refrigerator at $4^{0}$c until further analysis.

**HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY**

**Preparation of stock solutions of the four extracts of Clerodendrum serratum**

10mg of each extract was weighed and made up to 100ml with the respective solvents such as petroleum ether, chloroform, ethyl acetate and methanol. The concentration of 1000µg/ml solution were prepared and filtered over Whatmann filter paper. 20µg/spot of each solution was applied on the TLC plate.

**Preparation of stock solutions of the stigmasterol (Bio-marker)**

10 mg of marker compound was transferred into 10ml standard flask and was made with chloroform to get the concentration of 1000 µg/ml. 50 µg/ml solution was prepared from the above stock solution (300-900ng /spot; 3-15 µl/spot) was applied on the HPTLC plate.

**Preparation of stock solutions of the formulation**

The extracts were obtained from the formulation of *Clerodendrum serratum*.10mg of each extract was weighed, dissolved in methanol and made up to 100ml. From this solutions of 1000 µg/ml concentrations were prepared and filtered over Whatmann filter paper. 20 µg/spot of each solution was applied on the TLC plate. HPTLC is a sophisticated and automated form of TLC. HPTLC is the fastest of all chromatographic methods. Precoated, silica gel G 60 F25 (Merck, Germany) HPTLC plates were used for the application of sample.

**Fixed chromatographic parameters**

| | | |
|---|---|---|
| Plate | : | TLC aluminium sheets silica gel 60 F 254, [E.MERCK KGaA] |
| Plate size | : | 10×10 cm |
| Solvent | : | $CHCl_3 : CH_3OH$ (95:5 % v/v) |
| Instrument | : | CAMAG Linomat 5 "Linomat 100632" Linomat 5 applicator. |
| Wavelength | : | 366nm. |
| Detection | : | U.V. |

**Validation of HPTLC method**

The validation of the developed method was carried out in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision, repeatability of sample application, measurement, stability studies and selectivity.

**Linearity and Range**

Linear regression data showed a good linear relationship over a concentration range of 300 to 900ng/spot. The slope, intercept and correlation co-efficient values were found to be 0.9976.

**Accuracy**

Recovery studies of the drug were carried out for determining accuracy parameter. It was done by mixing known quantity of standard drug with the pre-analysed sample formulation and the contents were reanalysed by the proposed method. Recovery studies carried out at 80% and 100% levels. The percentage recovery and its % RSD were calculated and they are shown in table 4.

**Precision**

Precision of the method was demonstrated by-

i. Intra-day precision ii. Inter-day precision iii. Repeatability

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a) Repeatability of measurement b) Repeatability of sample application

**i) Intraday precision**

Intraday precision was found out by carrying out the analysis of the standard drug for two different concentrations in the linearity range of the drug for three times on the same day and %RSD was calculated.

**ii) Interday precision**

Inter day precision was found by carrying out the analysis of the standard drug for two different concentrations in the linearity range of the drug for two days and %RSD was calculated.

**iii) Repeatability**

**a)Repeatability of measurement**

Repeatability of measurement of peak area was determined by spotting 2µl and 4µl of formulation on pre-coated TLC plate. After development of the plate, the separated spots were scanned five times without changing position of the plate and %RSD was calculated.

**b)Repeatability of sample application**

Repeatability of sample application was assessed by spotting 2 and 4µl of the drug solution five times on pre-coated TLC plate followed by development of plate and % RSD was calculated.

**Limit of detection and limit of quantification**

LOD and LOQ were determined by applying decreasing amount of the drug in triplicate on the plate. The lowest concentration at which the peak is detected is called limit of detection and it was found to be 100ng/spot. The lowest concentration at which the peak is quantified is called limit of quantification and it was found to be 300ng/spot.

**Stability studies**

When the developed chromatographic plate is exposed to atmosphere, the analyte are likely to decompose. Hence, it is necessary to conduct stability studies. Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 6 hours as the reduction in peak areas was within the limits.

***Invitro* anti-oxidant studies of extracts of *Clerodendrum serratum* roots**

**Nitric oxide radical scavenging assay**

Various concentrations of the extract and sodium nitroprusside (5mM) in phosphate buffer saline (0.025 M, pH 7.4) in a total volume of 3 ml were incubated at room temperature for a period of 150 mins. After which, 0.5 ml of the incubated solution and 0.5 ml Griess reagent (1% sulphanilamide, 2% o-Phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) were added together and allowed to react for 30 mins. Control samples without the test compounds but with equal volume of buffer were prepared in a similar manner as was done for the test. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and successive coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm. The percentage inhibition of the extracts and standard were calculated. The experiment was carried out in triplicate using ascorbic acid (40-200 µg/ml) as positive control.

**% Scavenging Activity = [(Ac- As) / Ac] × 100**

Where, Ac is the absorbance of the control reaction and As is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC50. The IC50 value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of free radicals by 50%.

**RESULTS AND DISCUSSION**

Preliminary phytochemical studies revealed the presence of triterpenoids, tannins and glycosides in successive extracts. The extract of methanol showed more number of spots compared to other extracts in the preliminary TLC studies. Chloroform: methanol (9.5: 0.5 %v/v) was used as the mobile phase. The yield during extraction was highest in Petroleum ether extract which was found to be 13.81gm.

**HPTLC method**

For the determination of stigmasterol by HPTLC method different mobile phase systems were tried. A system comprising of chloroform: methanol (9: 0.5 %v/v) was selected because this system gave good separation with symmetric peaks, (Rf value: 0.80 ± 0.02) at a selected wavelength of 366 nm. Calibration curves were drawn with peak areas of standard drug versus concentration. Linearity was found over the concentration range of 300 to 900ng/spot (r=0.9967). After the development, the plate was stable upto 6 hours. Low relative Standard Deviation value showed that the developed method is precise. Limit of

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**Table 1: Amount of stigmasterol present in extracts**

| Extracts | Amount of stigmasterol present in 10mg of extract |
|---|---|
| Petroleum ether | 0.948 |
| Chloroform | 0.68 |
| Ethyl acetate | 0.23 |
| Methanol | 0.10 |
| Formulation | 0.761 |

**Table 2: Calibration data of stigmasterol (300-900 ng/spot)**

| Concentration(ng/spot) | Peak area |
|---|---|
| 300 | 2666.65 |
| 450 | 3755.30 |
| 600 | 4634.20 |
| 750 | 5450.18 |
| 900 | 6122.25 |

**Table 3: Validation studies:**

| Validation parameters | Volume applied (µl) | %RSD |
|---|---|---|
| **Intraday precision** | 3 | 0.7588 |
| | 6 | 0.4784 |
| **Interday precision** | 3 | 0.9687 |
| | 6 | 0.4550 |
| | 9 | 0.8151 |
| **Repeatability of sample injection** | 9 | 0.4514 |
| **Repeatability of measurement** | 6 | 0.6608 |

**Table 4: Recovery studies**

| Level | % Recovery | %RSD* |
|---|---|---|
| 80% | 102.98 | 0.96153 |
| 100% | 96.5 | 0.3751 |

* Mean RSD of three observations

**Table 5: % of maximum antioxidant activity of extracts**

| S. No. | Sample | % maximum Anti-oxidant activity [%] |
|---|---|---|
| 1 | Standard [Ascorbic acid] | 66.8828 |
| 2 | Petroleum ether extract | 52.5543 |
| 3 | Chloroform extract | 20.1656 |
| 4 | Ethyl acetate extract | 18.6795 |
| 5 | Methanol extract | 32.2787 |
| 6 | Formulation | 50.6587 |

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**Table 6: Nitric oxide scavenging activity control absorbance [Ac]: 1.002**

| Sample | Concentration (µg/ml) | Absorbance at 546 nm [As] | % Anti-oxidant Activity | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| Petroleum ether extract | 40 | 0.9980 | 10.2773 | 194.54 |
| | 80 | 0.9918 | 24.9550 | |
| | 120 | 0.9813 | 37.9465 | |
| | 160 | 0.8119 | 45.6795 | |
| | 200 | 0.9001 | 52.5543 | |
| Chloroform extract | 40 | 0.9915 | 1.0676 | – |
| | 80 | 0.9900 | 1.2173 | |
| | 120 | 0.8331 | 16.6872 | |
| | 160 | 0.8001 | 20.1656 | |
| | 200 | 0.8613 | 14.0590 | |
| Ethyl acetate extract | 40 | 0.8992 | 0.4773 | – |
| | 80 | 0.7521 | 1.0355 | |
| | 120 | 0.6219 | 2.9465 | |
| | 160 | 0.5444 | 18.6795 | |
| | 200 | 0.4755 | 10.5543 | |
| Methanol extract | 40 | 0.9050 | 9.6986 | – |
| | 80 | 0.8515 | 15.0369 | |
| | 120 | 0.8133 | 18.8485 | |
| | 160 | 0.7407 | 26.0925 | |
| | 200 | 0.6787 | 32.2787 | |
| Formulation | 40 | 0.8090 | 10.2773 | 192.54 |
| | 80 | 0.8545 | 22.5650 | |
| | 120 | 0.8113 | 36.9565 | |
| | 160 | 0.7707 | 43.6795 | |
| | 200 | 0.6687 | 51.5543 | |
| Standard [Ascorbic acid] | 40 | 0.7932 | 20.8541 | 198.98 |
| | 80 | 0.6139 | 38.7447 | |
| | 120 | 0.5311 | 47.0065 | |
| | 160 | 0.4793 | 52.1752 | |
| | 200 | 0.3319 | 66.8828 | |

**Fig.1: HPTLC chromatogram of Petroleum ether extract at 366nm**

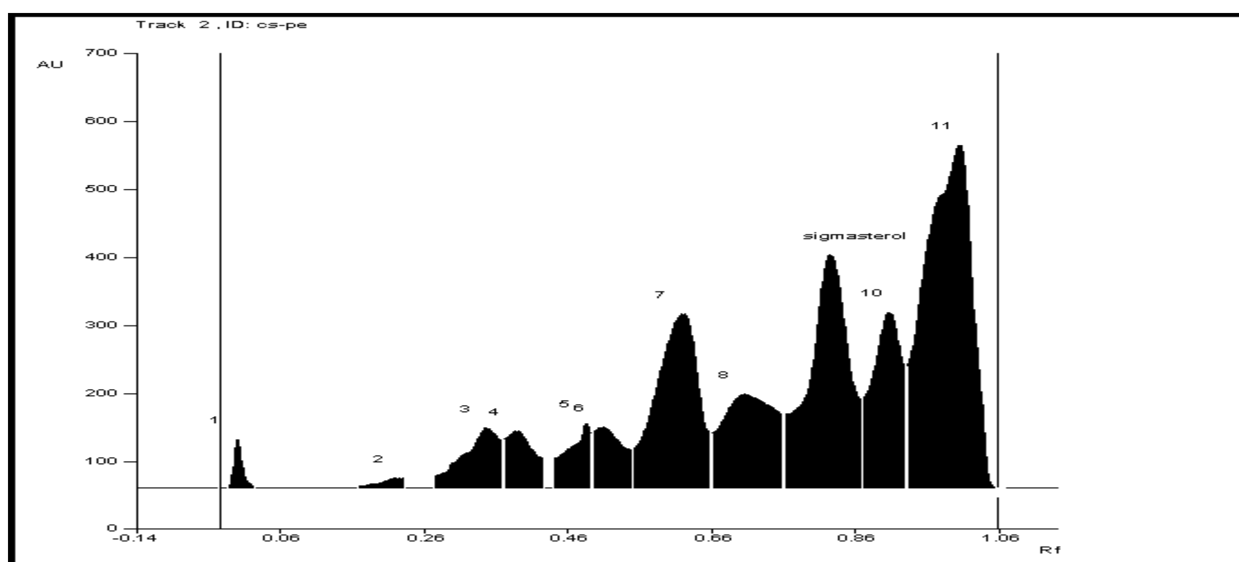


**Fig. 2: HPTLC chromatogram of Chloroform extract at 366 nm**

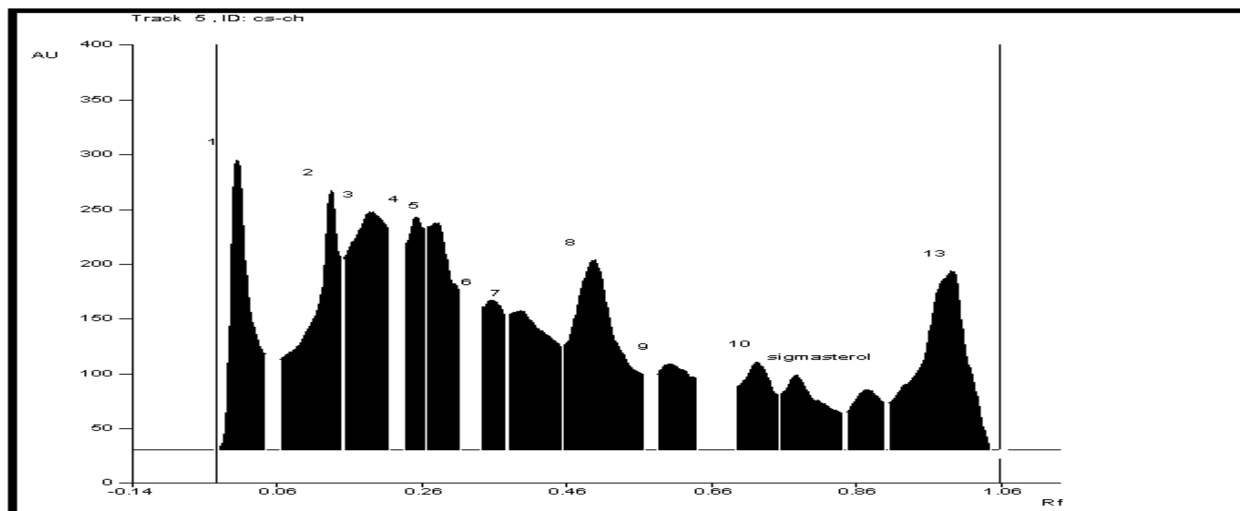



**Fig. 3: HPTLC chromatogram of Ethylacetate extractat 366 nm**

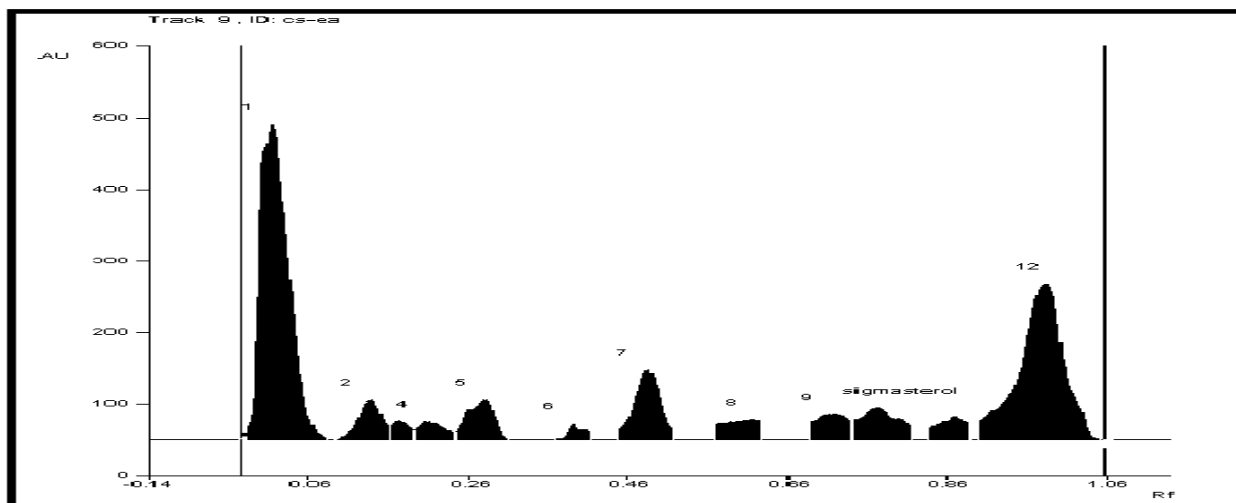



**Fig. 4: HPTLC chromatogram of Methanol extract at 366 nm**

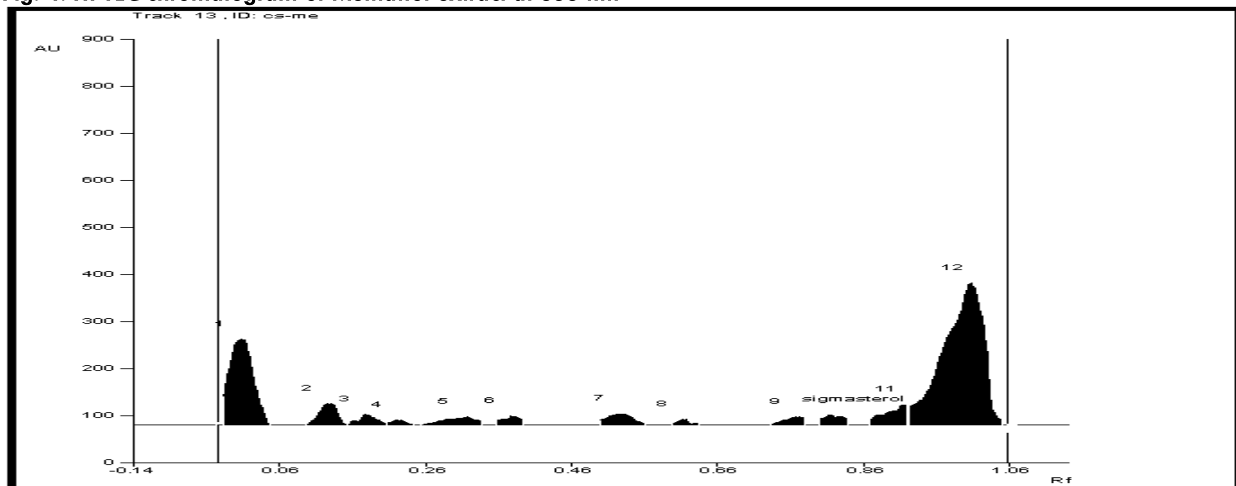


**Fig. 5: HPTLC chromatogram of formulation (supres)**

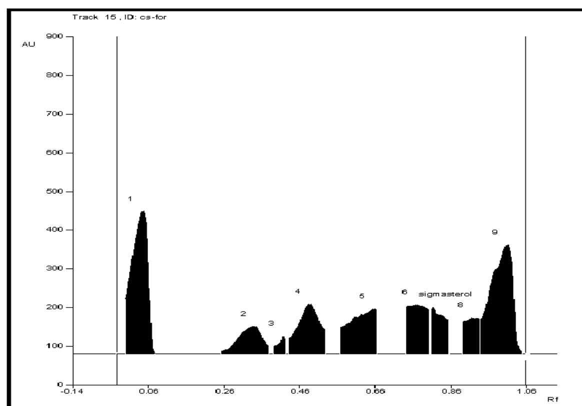
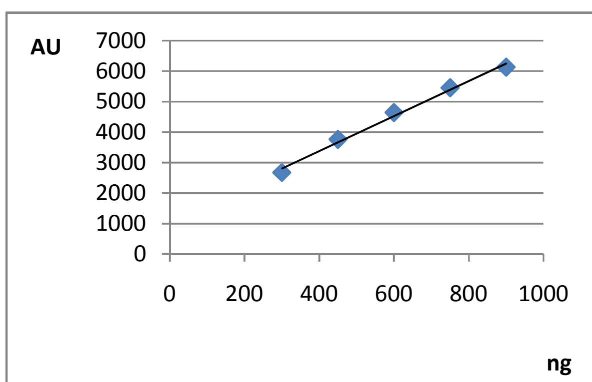


**Fig 6: Calibration graph of stigmasterol (300-900 ng/spot)**




detection was found to be 100 ng/spot and limit of quantification was found to be 300ng/spot. Results showed that more amount of stigmasterol present in petroleum ether when compared to other successive extracts and the polyherbal formulation. Recovery study was carried out at 80% and 100% levels. Good recovery values showed that the method is free from interferences. This method was successfully used for the determination of stigmasterol from polyherbal formulation.

***Invitro* antioxidant activity**

The antioxidant activity of the extracts of *Clerodendrum serratum* roots and polyherbal formulation were studied. The results showed that the polyherbal formulation containing stigmasterol has medicinal value and that it may be utillised for anti-oxidant properties.

**CONCLUSION**

The bark of *Clerodendrum serratum* contain high amount of triterpenoids, which is responsible for the main pharmacological actions like anti-oxidant, diuretic, anti-inflammatory actions etc. Therefore, the bark of *Clerodendrum serratum* had been selected for the present work. Most of the Ayurvedic formulations contain *Clerodendrum serratum* root powder or the extract of the bark powder as the main active portion in poly herbal formulations for their therapeutic action. One of the poly herbal formulations of *Clerodendrum serratum* was selected for the current study. HPTLC evaluation as recommended in the present study provides a chromatographic fingerprint of phytochemicals and is suitable for confirming the identity and purity of medicinal plant raw material. The anti-oxidant activity was highest in petroleum ether extract when compared to other extracts.

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