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Antioxidant activity, TLC and HPLC-ESI-Q-TOF-MS fingerprinting of *Catunaregam spinosa* (Thunb.) triveng

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Abstract

Catunaregam spinosa (Thunb.) Triveng belongs to family Rubiaceae and is known to possess anti-bacterial, anti-allergic, analgesic properties. Geographical location plays an important role in determining the phytochemical composition of the plant. Our aim was to determine differences in the bioactivity potential of the plant from Goa district when in comparison to the same from Mumbai. The leaf and stem of the plant from both regions were subjected to Anti-oxidant activity determination and TLC fingerprinting, followed by HPLC-ESI-Q-TOF-MS to determine probable compounds responsible for anti-oxidant activity. TLC showed differences in the fingerprints and the stem of *Catunaregam spinosa* (Thunb.) Triveng from Goa showed highest anti-oxidant activity with IC₅₀ value of <2mg/ml. HPLC-ESI-Q-TOF-MS of this sample revealed compounds such as Kaempferol, 4-Methylsculetin, Umbelliferone which have good anti-oxidant activity. This sample can further subjected to quantification and may be used in Herbal formulations.

Keywords: DPPH assay, flavonoids, sattari, Mumbai, coumarins

Introduction

Plants have been source of many active ingredients used in the drugs targeted against infectious diseases, many types of cancer, respiratory disorders etc. The secondary metabolites produced in different parts of the plant have potential therapeutic uses that have been applied in Ayurvedic and Allopathic formulations successfully. Many times, the availability of these sources, plants and trees, becomes limited due its location and abundance, and exhausting the plants from the same place endangers it's existence. Instead, the same plants may be present in a different geographical location from where they could be sampled easily. The important factor under consideration would be if the plant from different location would contain the same metabolites, with the same or better potency and bioactivity.

Many analytical and phytochemical techniques are available which aids in determining the differences in these plants at metabolite levels, for example, TLC, HPTLC, HPLC, quantitative phytochemical assays, Bioassays and some advanced instrumentation like LC-MS/MS, NMR, MALDI-TOF which can determine the quantitative differences of trace metabolites like flavonoids, polyphenols, tannins, terpenoids etc.

For the fruit of *Catunaregam spinosa* (Thunb.) Triveng to be used effectively in treatment of Jaundice by tribes in Sattari, Goa, it must have phytoconstituents that contributes towards it's bioactivity. *Catunaregam spinosa* (Thunb.) Triveng is a large shrub or small tree, which has been used in the treatment of various ailments like diarrhea, dysentery, antipyretic, wounds, tumors, skin diseases^[1,2] roots are used for treatment of epilepsy, urinary infection; leaves are used to treat pulmonary infection^[3,4]. It has been used in formulation of medicines in Ayurveda, Siddha, Unani, folk and Sowa-Rigpa, and has been listed as "Madana" or "Madanphal" under Ayurveda^[5]. Thin layer chromatography is an analytical tool that allows for identification and authentication of natural products. It separates compounds in the plant extract that allows for identification of thousands of compounds. This technique can be used for comparison of very similar samples^[6]. TLC has been used extensively to study differences in plant extracts from different geographical locations. Quantitative TLC to find differences in Ascorbic acid levels of different varieties of *Phyllanthus emblica* Linn from Shirpur and Mumbai was performed, which showed highest level in bigger variety from Shirpur^[7]. In another study where "Propolis" collected from different regions in Central and South Eastern Europe showed differences in flavonoid and phenolics pattern, which could be attributed to climatic conditions and soil characteristics^[8]. "Bacoside A", a triterpenoid isolated from *Bacopa monnieri* (L). Wettst from different geographical regions in India, showed highest quantity from Jammu and least from Kerala as assessed by HPTLC^[9].

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Anti-oxidant property is very important to prevent cell damage and is found in naturally occurring food. This property can be correlated to the total phenolic and flavonoid content in the plant. Higher the phenols, higher is the anti-oxidant activity. The total phenol content is dependent on the environmental conditions specifically the nutrients obtained from soil. Comparative in- vitro antioxidant activity of flaxseed from different geographical origin in China showed variation in the activities [10]. Another such study of antioxidant activity of *Ruta chalepensis* leaf essential oils collected from three different Palestinian regions showed least IC50 value in the sample from Jerusalem as compared to other two [11]. This property is present in various parts of the plant and can be assessed by DPPH assay, FRAP assay, ABTS assay etc. HPLC- ESI- Q- TOF-MS allows for identification of compounds present in the sample. Liquid Chromatography separates the compounds in the column and the individual components then enters ESI-Q-TOF-MS where it's characteristics such as molecular weight, m/z, abundance in the sample is detected. This method has been employed to analyze plant extracts for detection and quantification of phenols, flavonoids, terpenoids, peptides etc.

Catunaregam spinosa (Thunb.) Triveng leaf and stem were sampled from ethnobotanical study site- Sattari, Goa and metropolitan city- Mumbai for the comparative TLC and anti-oxidant study. To study the differences in metabolites, Thin layer chromatography was employed. To assess the components responsible for anti- oxidant activity in the Mumbai stem sample, HPLC- ESI-Q-TOF-MS method was employed.

Materials and Methods

1. Sample Preparation

The leaves and the stem of plants were separated and air dried following which it was oven dried at 55°C till a constant weight was obtained. The samples were ground and stored in dark conditions. The powdered samples were then subjected to maceration with HPLC Grade Methanol, for 2 hours at RT on Shaker, following which the supernatant was separated and used for different experiments.

2. Antioxidant activity by DPPH assay

To determine the antioxidant activity 0.5g of the dried powdered sample was macerated with 5ml of Methanol for 2 hours at RT on shaker after which the supernatant was used for estimation. Ascorbic acid was used as standard at concentrations ranging from 2mg/ml to 10mg/ml. Similarly, the same concentration of test sample was prepared in

methanol. To 0.2ml of each standard and sample, 3.8ml of 0.1mM DPPH reagent in methanol was added. The tubes were vortexed vigorously and incubated at RT in dark for 30 minutes. The absorbance of the reaction mixture was measured at 517nm using UV Visible spectrophotometer. A graph was then plotted in which the samples were plotted along with the standard vs Percentage Inhibition [12]

3. Thin Layer Chromatography

TLC plates Silica Gel 60F 254 were used; 2µl and 5µl of 100mg/ml methanolic extract of stem and leaf was applied on the plates using Linomat- 5 applicator. The plates were then developed in Toluene: Chloroform: Ethanol (4:4:1) mobile phase till the solvent reached 70mm of the plate. The plates were dried using hair dryer, observed and scanned under white light and UV (254nm). The plate was derivatized using Natural Product reagent and scanned under 366nm. This was followed by derivatization with 10% Methanolic Sulfuric Acid. The plates were scanned at 366nm and white light. The compounds are recorded as peaks on the chromatogram by Densitometer.

4. HPLC- ESI-Q-TOF-MS

HPLC- ESI-Q-TOF-MS analysis of methanolic stem extract of *Catunaregam spinosa* (Thunb.) Triveng from Sattari, Goa was performed on Agilent Technologies 6550 iFunnel Q-TOF LC/MS which included an Agilent 1290 Infinity UHPLC having Binary pump, Thermostatted column compartment, Autosampler, Thermostat and Jetstream. A two- solvent system was used which was as follows: Solvent A: Water+ 0.1% Formic acid & Solvent B: 90% Acetonitrile+ 10% water+0.1% Acetonitrile; a gradient started with 95% Solvent A and ended with 100% Solvent B. The flow rate was maintained at 0.3ml/min and injection volume was 3µl. The column used was C18 column and the column outlet was connected to Mass spectrometer via Dual AJS ESI. Both Positive and Negative modes of ESI was used to Ionize the compounds which was channelized using Quadrupole to TOF. A photomultiplier plate was used to detect the m/z values of the ionized compounds. The MS spectra obtained of the analyzed sample was searched against Metlin database to find the probable compounds present in the sample.

Results

1. Antioxidant activity

The DPPH assay performed showed increasing anti- oxidant activity with increasing concentration of plant extract. Percentage of inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100 \%$$

The values were plotted on a Concentration of plant extract vs Percentage Inhibition Graph. The IC50 values were calculated from the graph. The *Catunaregam spinosa* (Thunb.) Triveng Stem sample from Sattari, Goa shows most potent anti-

oxidant activity as it shows IC50 at a least concentration of less than 2mg/ml. IC50 values for other samples are as follows:

Table 1: IC50 values of the Stem and Leaf samples of *Catunaregam spinosa* (Thunb.) Triveng

Plant sample	Ascorbic acid	CSMS	CSGS	CSML	CSGL
IC50 value (mg/ml)	<2	8.08	<2	5.35	9.5

Key: CSMS- *Catunaregam spinosa* Mumbai Stem, CSGS- *Catunaregam spinosa* Goa Stem, CSML- *Catunaregam spinosa* Mumbai Leaf, CSGL- *Catunaregam spinosa* Goa Leaf

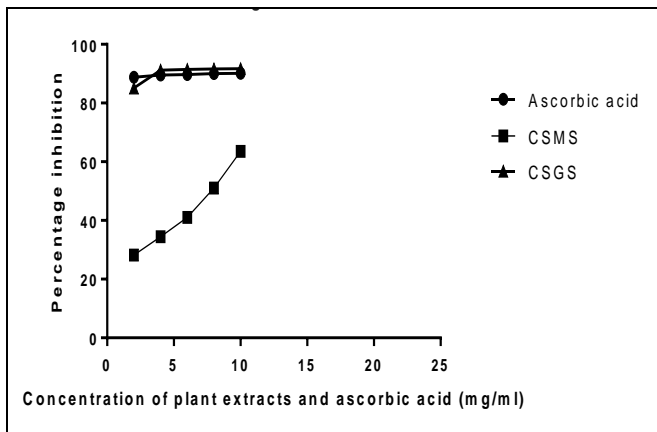


Fig 1: Comparative Anti-oxidant activity of Stem samples of *Catunaregam spinosa* from Sattari, Goa and Mumbai (CSMS- *Catunaregam spinosa* Mumbai Stem, CSGS- *Catunaregam spinosa* Goa Stem)

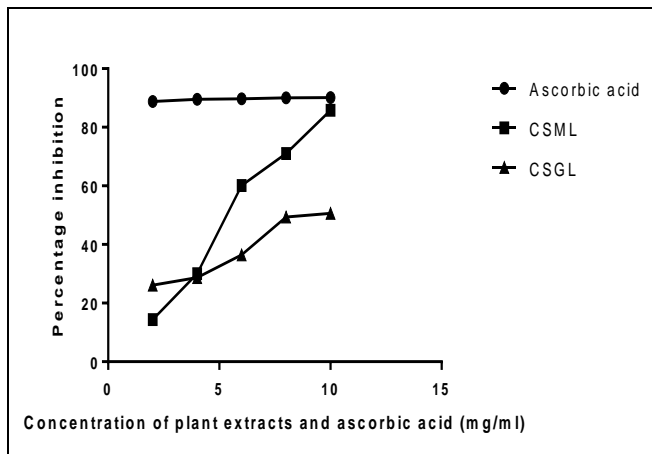


Fig 2: Comparative Anti-oxidant activity of Leaf samples of *Catunaregam spinosa* from Sattari, Goa and Mumbai (CSML- *Catunaregam spinosa* Mumbai Leaf, CSGL- *Catunaregam spinosa* Goa Lea)

2. Thin layer Chromatography

TLC of Leaf and Stem samples showed differences in the number of compounds present under different wavelengths.

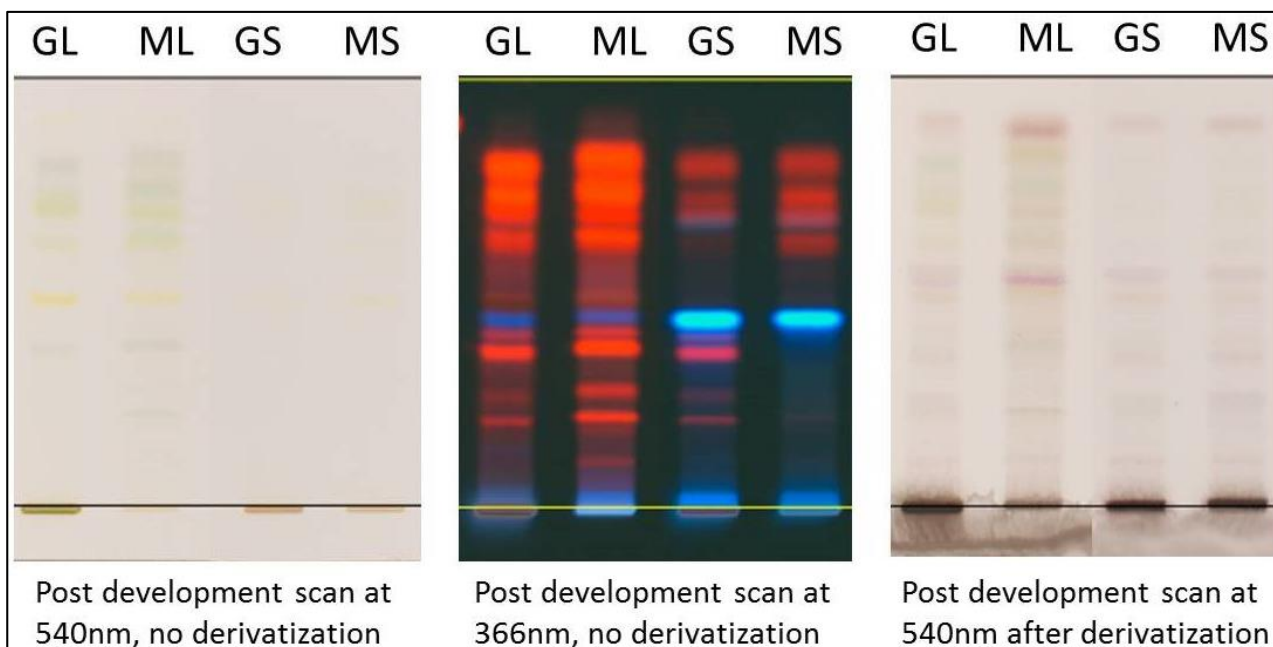


Fig 3: Images of TLC Chromatogram of Leaf (GL- Goa Leaf, ML- Mumbai Leaf) and Stem (GS- Goa Stem, MS- Mumbai Stem) under different wavelengths

Significant differences in the number of bands corresponding to number of compounds present were observed underivatized

under 254nm and derivatized under 366nm. The chromatograms are as follows:



Fig 4: Image of TLC Chromatogram underivatized under 254nm

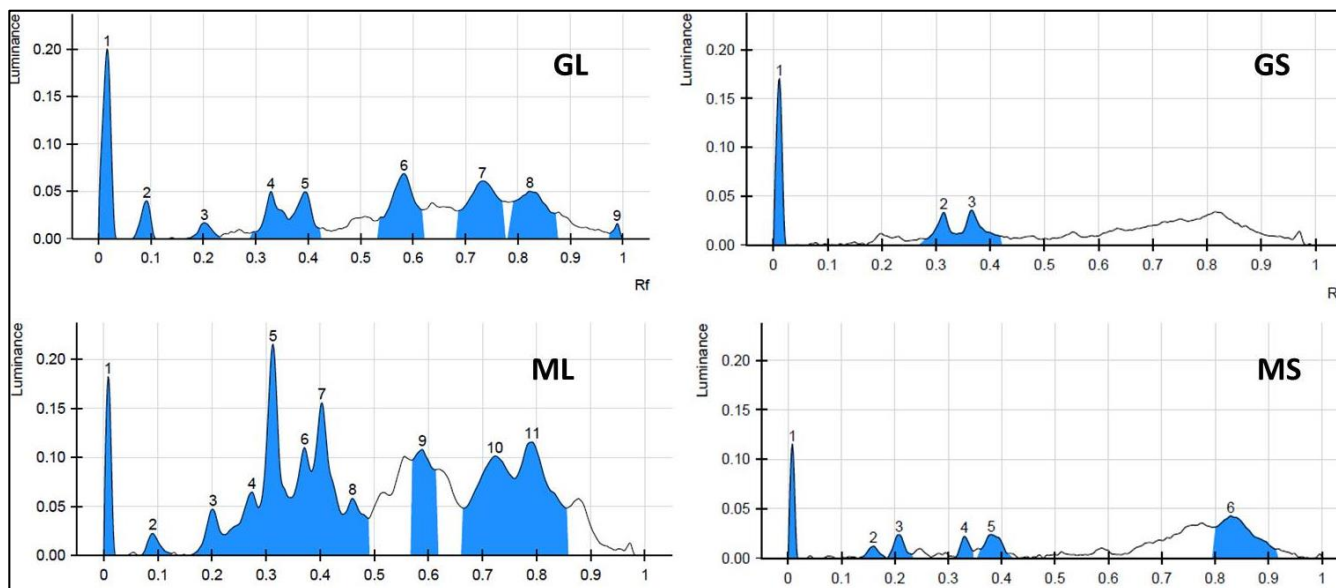


Fig 5: Graphs of respective TLC scans under 254nm, underivatized

From the above scans, 9 peaks from Goa Leaf, 11 peaks from Mumbai Leaf, 3 peaks from Goa Stem and 6 peaks from Mumbai Stem were recorded underivatized under 254nm. Leaves from Goa and Mumbai showed 2 sets of peaks with similar Rf values, viz., 0.063 & 0.068; 0.289 & 0.285 respectively. Stem from Goa and Mumbai also showed 2 sets of peaks with similar Rf values, viz., 0.281 & 0.287; 0.345 & 0.345 respectively.

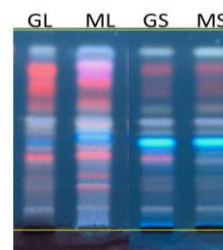


Fig 6: Image of TLC Chromatogram derivatized by 10% methanolic sulphuric acid scan under 366nm

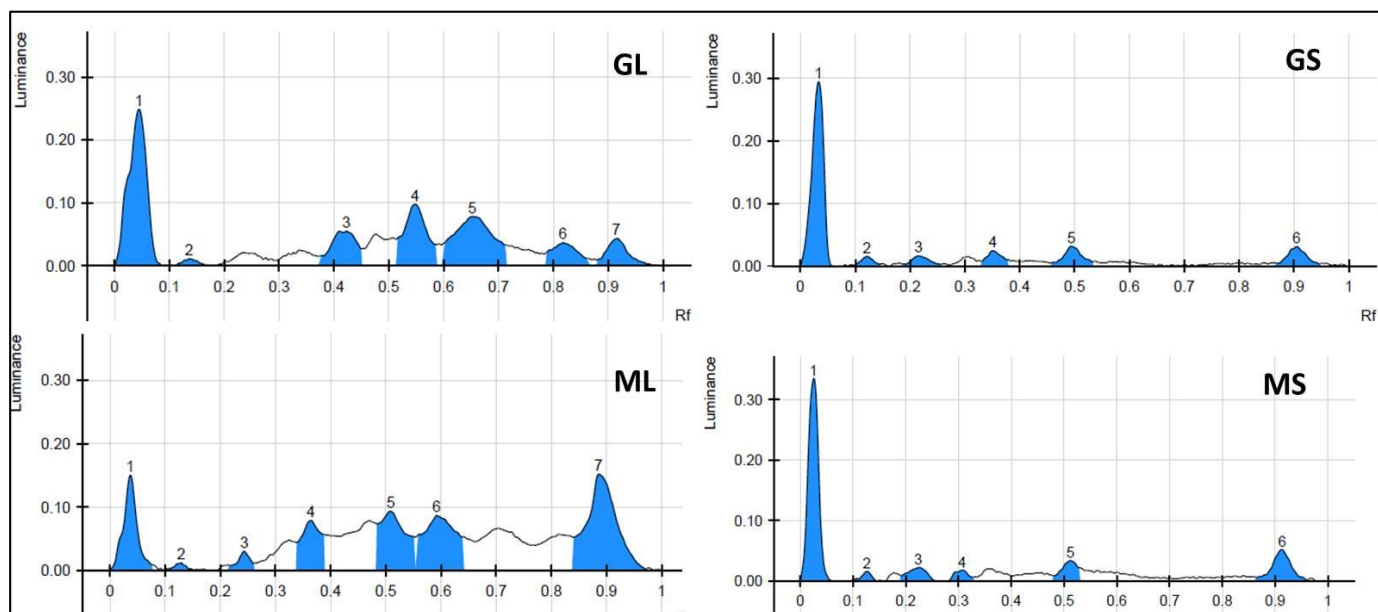


Fig 7: Graphs of respective TLC scans under 366nm, derivatized

From the above derivatized scan 366nm, 7 peaks in Goa and Mumbai Leaf, 6 peaks in Goa and Mumbai stem were observed respectively. Derivatization with 10% methanolic sulphuric acid aided in detecting compounds, especially in stem samples, which otherwise remained undetected. Leaves from Goa and Mumbai showed only one set of similar peak, viz., 0.136 & 0.129. On the other hand, Stem from Goa and Mumbai showed 3 sets of similar peaks, viz., 0.121 & 0.126; 0.216 & 0.224 and 0.905 & 0.913.

3. LC-MS/MS Analysis

Catunaregam spinosa (Thunb.) Triveng Stem sample from Goa showed highest antioxidant activity, with least IC50 value of <2mg/ml. Its methanolic extracts were further subjected to LC-MS/MS analysis to find probable compounds responsible for its antioxidant activity. Compounds responsible for antioxidant activity were found to be present in the extract.

Table 2: Important compounds identified qualitatively in methanolic stem extracts of *Catunaregam spinosa* (Thunb.) Triveng from Goa by LC-MS/MS

Label	Compound Name	Retention time	Mass	Formula	Abundance	Mode of Ionization
8	4-Hydroxylevamisole Glucuronide	2.712	396.1012	C17 H20 N2 O7 S	393018.69	+ESI
11	Citropten	3.135	206.0569	C11 H10 O4	449256.38	+ESI
12	Juglone	3.136	174.0307	C10 H6 O3	273211.13	+ESI
15	Sulfinpyrazone	3.199	404.1296	C23 H20 N2 O3 S	1352269	+ESI
20	Mono-N-hydroxydapsone	4.08	264.0598	C12 H12 N2 O3 S	282605	+ESI
29	Umbelliferone	5.356	162.0308	C9 H6 O3	1338423.25	+ESI
30	Chlorogenic acid	5.359	354.0934	C16 H18 O9	757128.88	+ESI
33	4-Methylesculetin	6.574	192.0411	C10 H8 O4	7653721	+ESI
34	Kaempherol	6.598	286.0463	C15 H10 O6	389540.94	+ESI
37	Irigenin, Dibenzyl Ether	7.047	540.1796	C32 H28 O8	645644.69	+ESI
55	3,4,5-Trihydroxystilbene	13.321	228.0775	C14 H12 O3	428210.41	+ESI
5	Ceftibuten	2.551	410.039	C15H14 N4 O6 S2	221131.25	-ESI
9	Leoidin Dimethyl Ether	3.804	440.048	C20 H18 Cl2 O7	739447.13	-ESI
13	Xipamide	5.003	354.0411	C15H15ClN2O4 S	887581.19	-ESI
23	Moxalactam	5.884	520.093	C20 H20 N6 O9 S	310404.41	-ESI
49	Praziquantel	14.495	312.1792	C19 H24 N2 O2	508778.09	-ESI
50	Desmethyltrimipramine glucuronide	16.138	456.2215	C25 H32 N2 O6	190633.27	-ESI

Discussion

Leaf and Stem samples of *Catunaregam spinosa* (Thunb.) Triveng from Sattari and Mumbai were subjected to comparative antioxidant activity assay and Thin layer chromatography. From the results, a significant difference in the antioxidant activities between samples from Sattari and Mumbai and between Leaf and Stem samples can be observed. The differences lies in the types and quantity of metabolites contributing to this activity to a great extent, for example, polyphenols, flavonoids etc., The micronutrients required for the production of the metabolites comes majorly from the soil, hence it is necessary to estimate it in the soil. It probably varies from place to place, indirectly contributing to the plant's antioxidant activity.

Thin layer chromatography showed maximum number of compounds to be present in the Leaf sample of *Catunaregam spinosa* (Thunb.) Triveng from Mumbai. In all samples a compound is commonly present at Rf value 0.28 underivatized as observed under 254nm and at Rf value 0.13 derivatized as observed under 366nm. This compound may serve as a phytochemical marker for *Catunaregam spinosa* (Thunb.) Triveng plant from any geographical location, different formulations and drugs. 10% methanolic sulphuric acid efficiently derivatized undetectable compounds. TLC can further be performed to determine the number of compounds present in each class of compounds in every sample using respective derivatizing agent.

Catunaregam spinosa (Thunb.) Triveng stem sample from Goa showed highest antioxidant activity as compared to other samples. HPLC-ESI-Q-TOF-MS analysis of this sample revealed presence of important secondary metabolites and also some antimicrobial compounds. Chlorogenic acid, 4-Methylesculetin, Umbelliferone, Kaempherol and 3,4,5-Trihydroxystilbene are known have very good antioxidant activity [13-17]. Of these metabolites, most abundantly present in the sample were 4-Methylesculetin and Umbelliferone. Coumarins have been previously reported to be present in *Catunaregam spinosa* (Thunb.) Triveng, along with Iridoids and Triterpenoids [18-19]. Apart from these, other compounds such as Leoidin & Irigenin are natural metabolites that are antimicrobial and antifungal respectively [20, 21]. Structures similar to Ceftibuten, Mono-N-hydroxydapsone and Moxalactam were seen to be present, which are reported to have antimicrobial activity [22-24]. The above reported compounds are being reported for the first time in

Catunaregam spinosa (Thunb.) Triveng. Further, characterization by NMR and quantification of the above mentioned compounds by Mass spectrophotometer can be carried out to establish the fingerprint. The LC-MS/MS studies suggest that this plant possesses potential antioxidant and antimicrobial compounds, whose production can further be traced upto molecular level.

Comparative study of *Catunaregam spinosa* (Thunb.) Triveng stem and leaf from Sattari (Goa) and Mumbai was carried out by antioxidant activity assay and TLC fingerprinting studies. Differences in results were observed which can be attributed to differences in climate, air, temperature, humidity and soil nutrients availability. Similarity observed in presence of certain compounds by TLC can be used as a marker. HPLC-ESI-Q-TOF-MS analysis of stem sample from Sattari reveals potential bioactive compounds that can further be characterized. It can therefore be used in formulation of pharmaceutical drugs and other herbal products.

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