IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN(E): 2321-8851; ISSN(P): 2347-4580
Vol. 3, Issue 5, May 2015, 9-26

PRELIMINARY SECONDARY METABOLITES SCREENING AND GC-MS ANALYSIS OF PLANT EXTRACT OF *TRIDAX PROCUMBENS* (L)

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ABSTRACT

Tridax procumbens Linn. is a weed found throughout India. It is reported to be composed of useful phytochemicals for medicinal purpose. In the present study, preliminary phytochemical screening of *T. procumbens* stem, leaf and callus cultures were carried out. Using TLC presence of two Sterols like β -sitosterol, stigmasterol and three flavonoids viz., Kaempferol, Quercetin and Luteolin, were identified. They were evaluated by their color, RF value, TLC behavior, melting point, and IR spectral studies Quantitative analysis revealed the presence of 5.88 mg/g.dw of total sterols, and 3.51 mg/gdw of total flavonoids. GC-MS analysis detected the presence of 33 useful compounds. In the leaf extract highest peak area (%) 14.65, was obtained by ethyl oleate and the lowest peak area (%) 0.46 was obtained by 9-Octadecenamide. Whereas, in stem extract the highest peak area (%) 14.24 was obtained by Tetracontane and the lowest peak area (%) 0.49 was obtained by 2, 8-Dimethyl-2-(4, 8, 12-Trimethyltridecyl)-6-CH. The total ion chromatogram showing the peak identities of the compounds have been identified using NIST library in the plant species. The presence of various bioactive compounds justifies the use of this plant in the modern system of health care for developing a novel drug.

KEYWORDS: *Tridax procumbens*, TLC, GC-MS, Flavonoids, Sterols

INTRODUCTION

Plants are predominant part of our everyday diet, their ingredients and nutritional values have been extensively studied for decades. Higher plants apart from the essential primary metabolites (e.g. carbohydrates, lipids, protein and amino acids) also synthesize a wide variety of low molecular weight compounds that is, the secondary metabolites. Plant secondary metabolites can be defined as compounds, that have no specific role in the maintenance of fundamental life processes in the plants but they do have an important role in the interaction of the plants with its environment (Sirikantaramas *et al.*, 2008). Secondary metabolites play a major role in the adaptation of plants to the changing environment and in overcoming stress constraints.

Secondary metabolites are the basic source for the establishment of pharmaceutical industry, food, perfumes, growth and morphological differentiation of cells (Bell, 2001, Vanisree and Hsin-sheng, 2004). Some secondary compounds produced in plants are important to protect these plants against microorganism and other plant species for ecological habits. (Stojakowsk and Kisiel, 1999). Essential class of secondary compounds include organic acids (aliphatic, aromatic and heteroaromatic), phenolic compounds (simple phenols and their products, phenol glycosides, phenol acids, phenylnpropanoids, coumarins, xanthones, flavonoids, gibbrellins, triterpenes, carotenoids, polyterpenes), saponins (steroid

saponins, triterpenoid saponins), alkaloids and other nitrogenous compounds (amines, cyanogenic hetersides) (Mulabagal et al., 2004; Cervia et al., 2009; Thilagavathi et al., 2010;Savitharamma et al.,2011).

Tridax procumbens L. belonging to family Asteraceae is a common medicinal herb used by ethno-medical practitioners. It is well known as a widespread weed and pest plant. It is native to tropical America but it has been spread to tropical, subtropical and mid temperate regions worldwide. The plant is a procumbent herb and is valued for its pharmaceutical properties. (Sahoo and Chand, 1998). It has been found to possess significant medicinal properties against stomach ache, diarrhea, dysentery, blood pressure, malaria, bronchial catarrh, wound healing, headache etc. It also prevents hair fall and check hemorrhage from bruises and cuts (Ali et al., 2001). Its flowers and leaves possess insecticidal, antiseptic, and parasiticidal properties (Sahoo and Chand ,1998), (Pathak et al., 1991). The plant also shows various pharmacological activities like Anti hepatotoxic, Anti-inflammatory, Immunomodulatory, Analgesic, Antidiabetic, & Anti-oxidant, and significant depressant action on respiration (Ravikumar et al., 2005), (Vyas et al., 2004), (Bhagwat et al., 2008), (Redipalli,2008), (Diwan et al, 1989), (Jain, 2006). The plant has been reported extensively as antimicrobial, antidiabetic, anti-inlammatory, analgesic and anticancerous agent (Jain and Jain, 2012). Essential biomolecules like catachol, phenolic compounds, steroids, anthraquinone, flavonoids, terpenoids, saponins, and tannins were revealed by qualitative analysis. The studies on plant *Tridax procumbens* also desire development of novel therapeutic agents from the various types of compounds with diverse pharmacologic properties isolated from it. As there is lot of potential of this plant for its pharmacological activity, in the present study various secondary metabolites from *T .procumbens* were extracted and characterized both qualitatively and quantitatively. Moreover identification of essential compounds from methanolic and benzene extracts of leaf, stem respectively, of *T. procumbens* by GC-MS analysis was carried out.

MATERIALS AND METHODS

During the present study β-sitosterol, stigmasterol, quercetin, kaempferol and luteolin were isolated from leaves, stem and callus of *Tridax procumbens.*

Essential Chemicals

(i) Solvents: Hexane, Petroleum ether, benzene, chloroform ethylacetate, acetone, methanol etc. (ii) 15% ethanolic HCl (iii) Anhydrous sodium sulphate solution (3%) (iv) Silica gel (G)

Plant Material

(i) Leaves and stem of *T. procumbens* were collected from University of Rajasthan, Jaipur. The plant was taxonomically identified and authenticated *(*RUBL211384) by herbarium of Rajasthan University, Jaipur. (ii) For *in vitro* studies nodal segments as explants were used to initiate callusing. Explants presoaked in 0.1% liquid detergent for 30 minutes, were washed with running tap water and then surface sterilized with 0.5% (w/v) mercuric chloride for 3 min followed by two or three rinses of sterile distilled water. Murashige and Skoog's medium (1962) supplemented with various concentrations and combinations of growth hormones were used. Callus was maintained for six months by frequent sub-culturing at interval of 6 to 8 weeks at 26 ± 1 ^{o}C, 60% relative humidity and diffused light conditions (3000 lux). Growth Indices (GI) of tissue was calculated at 2, 4, 6, 8 and10 weeks time intervals. These callus tissues obtained were separately dried, powdered and used for flavonoid and steroid extraction.

Extraction of Flavonoids

Different plant parts (leaves, stem) along with callus tissue of *T. procumbens* were air dried and powdered, separately. Each of these tissues was extracted separately with 80% methanol on water bath (Subramanian & Nagarajan, 1969) for 24 h. The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fr1), diethyl ether (FrII) and ethyl acetate (FrIII) separately. Each step was repeated thrice for complete extraction. Fraction 1 was discarded in each case as it contained fatty substance, where as fraction II and fraction III were concentrated and used for determining flavonoids.

Fraction III was further hydrolyzed by refluxing with 7% sulphuric acid (10 mlg^{-1} plant material for 2 h), filtered and filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo*. Both fraction II and III were taken up in small volume of ethanol (2-5ml) before chromatographic examination.

Qualitative Analysis

Thin Layer Chromatography (TLC)

Thin glass plates (20 x 20 cm) were coated with Silica gel G (250 mμ thick). The freshly prepared plates were air dried at room temperature; thereafter these were kept at 100 ^{o}C for 30 min to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis.

Each of the extracts was co-chromatographed with authentic flavonoid as a marker (quercetin, luteolin, kaempferol). These plates were developed in an air tight chromatographic chamber saturated with solvent mixture (Benzene: Acetic Acid: Water:: 125:72:3; Wong & Francis, 1968). The developed plates were air dried and visualized under UV light by exposure to ammonia fumes. The mouth of a 100 ml containing concentrated NH_4OH was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The developed plates were also sprayed with 5% $FeCl_3$, 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber separately. The colored spots thus developed were noted and the Rf value of each spot was calculated. Several others solvent systems such as n-butanol, acetic acid, water (4:1:5), tertiary butanol, acetic acid, water (3:1:1) were also tested.

Identification

The identity of the isolated flavonoids were confirmed by mp, mmp performed in capillaries (Toshniwal Melting Point Apparatus), IR (Infra-red spectrophotometer; Perkin, Elmer 337, Grating Infra-red spectrophotometer), UV (Ultraviolet and visible spectrophotometer; Carl Zeiss, VSU-2P spectrophotometer) analysis along with their respective authentic samples.

Quantification

The isolated flavonoids were estimated by spectrophotometer following the method of Mabry et al .,(1970). Stock solution (1 mgl^{-1}) of kaempferol, luteolin, quercetin were prepared separately by dissolving authentic compounds in methanol. Different concentrations ranging from 20 μg to 160 μg of each of the compounds spotted separately on silica gel G plates. For each concentration of reference authentic standards separate plates were used and developed in the same manner as described earlier. These developed plates were air dried and visualized under UV light. The fluorescent spots

were marked and collected along with the absorbance in separate test tubes. Spectroscopy methanol grade (5ml) was added to each test tube, shaken vigorously, centrifuged and supernatants were collected separately. The volume of each of the eluate was made up to 10ml by adding methanol. To each of these samples, 3ml of 0.1 M $AlCl_3$ solution was added again shaken vigorously and kept at room temperature for 20 min. Five such replicates were run in each case and their optical densities were measured using spectrophotometer at 426 nm for kaempferol and luteolin and at 440 nm for quercetin against blank (10ml of spectroscopic grade methanol and 3 ml of 0.1 M $AlCl_3$). The standard curves were plotted between concentration and their respective average optical density of each of the compound. The regression curve so achieved followed Beer's law.

Each of the plant extract sample (ether and ethyl acetate sample) was dissolved in 5 ml of spectroscopic grade methanol and 0.1 ml was applied on silica gel G coated plates along with standard markers, separately. The plates were developed as above and the spots coinciding with that of standard markers were marked on each plate under UV. Each spot was collected along with the silica gel, eluted in methanol and test samples were prepared in the same way as described above. The optical density in each case was recorded and concentration of each sample was computed using the regression curve of authentic flavonoids samples. The concentrations were calculated on mg/g dry weight basis.

Extraction of Steroids

Different plant parts of *T. procumbens* (leaves, stem and callus tissue) were dried, powdered, weighed and defatted separately in soxhlet apparatus in petroleum ether for 24 h on a water bath. Each mixture was hydrolyzed with 15% ethanolic HCl (1g/5 ml: w/v) for 4 h by refluxing on water bath (Tomita et al., 1970). Each hydrolysate was filtered and filtrate was extracted thrice with ethyl acetate. The ethyl acetate fractions of each sample was pooled and washed repeatedly with distilled water to neutrality, dried *in vacuo*, reconstituted in chloroform, filtered, dried again and weighed. Each test sample was replicated thrice. Thin glass plates coated with silica gel (250mμ thick) were dried at room temperature, thereafter kept at 100^{o}C for 30 min to activate. The freshly prepared activated plates were used for qualitative as well as quantitative analysis.

Qualitative Analysis

The crude steroidal extract of each sample was examined on TLC, along with the reference steroids. The plates were developed in a solvent system of chloroform, hexane and acetone (23:5:2), air dried and sprayed with 50% sulphuric acid (Bennett & Heftmann, 1962) and anisaldehyde reagent (composed of 0.5 ml of anisaldehyde, 1 ml of conc. sulphuric acid and 50 ml of acetic acid) separately and heated to 100 ^{o}C until the characteristics colors develop. The fluorescence response as well as permanent black zones was recorded. The time required for the initial appearance of a color reaction, the initial color in day light and after heating for 10 minutes and the color in UV light (360 nm) was recorded. A combination of other solvent systems such as benzene and ethyl acetate (85:15; Heble et al., 1968) and acetone and benzene (1:2; Khanna & Jain, 1973) were also used. Three replicates were run and Rf values were calculated.

Quantitative Analysis

Preparative thin layer chromatography (PTLC)

PTLC was used to isolate steroids from crude steroidal extract on silica gel G plates by using solvent mixtures of chloroform, hexane and acetone (23:5:2). The spots were marked on TLC by spraying with anisaldeyde reagent, to one of

the columns on each plate and spots corresponding to the standard were marked and scrapped separately from the unsprayed plates/column. The PTLC was repeated until about 20 mg of the substance was obtained. Such chromatograms were also visualized by spraying a solution of aluminium trichloride in conc. HCl (Kadkade et al, 1976) and examined for mp, mmp and infra –red spectral studies.

Identification

The position of each component of a mixture is quantified by calculating the distance travelled by the component relative to the distance travelled by the solvent. This is called relative ratio of flow and symbolized by R_f value of each component, which can be determined by measuring the distance travelled by each component (solute) and the distance travelled by the solvent from the origin. The R_f value is calculated using the formula. (Heble *et al.*, 1968).

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

GC-MS Analysis

Preparation of Sample for GC-MS Study

About 20 g of the leaf and stem was powdered and were soaked in 100 ml methanol and benzene respectively. It was left for 24 h so that alkaloids, flavonoids, terpenoids and other constituents if present get dissolved. The extract was filtered using Whatman No.1 filter paper and the residue was removed. It was again filtered through sodium sulphate in order to remove the traces of moisture.

GC-MS Analysis

The GC-MS analysis of the plant extract was made in a (QP 2010 plus Shimadzu) instrument under computer control at 70 eV. About 1µl of the extract was injected into the GC-MS using a micro syringe and the scanning were done for 45 min. As the compounds were separated, they eluted from the column and entered a detector which was capable of creating an electronic signal whenever a compound was detected. The greater the concentration in the sample, bigger was the signal obtained which was then processed by a computer. The time from when the injection was made (Initial time) to when elution occurred is referred to as the Retention time (RT). While the instrument was run, the computer generated a graph from the signal called Chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the Gas chromatography column into the detector. The X-axis showed the RT and the Y –axis measured the intensity of the signal to quantify the component in the sample injected. Before analyzing the extract using oven, the flow rate of the gas used and the electron gun were programmed initially. The temperature of the oven was maintained at 100 ^{o}C. Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set to 1ml per minute. The electron gun of mass detector liberated electrons having energy of about 70eV. The column employed here for the separation of components was Elite 1 (100% dimethyl poly siloxane).

As individual compounds eluted from the Gas chromatographic column, they entered the electron ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass. The M/Z (Mass/Charge) ratio obtained was calibrated from the graph obtained, which was called as the Mass spectrum graph which is the fingerprint of a molecule.

RESULTS

In the present study, steroid and flavonoid profile has been qualitatively and quantitatively analyzed in *in-vivo* and *in-vitro* plant samples of *T. procumbens*.

Qualitative Analysis by thin Layer Chromatography

In the present study, TLC profiling of the plant extracts in different solvent system confirms the presence of various phytosterols. Various phytosterols gives different Rf values in different solvent system. This variation in Rf values of the phytosterols provide a very important clue in understanding of their polarity. The identified sterols were evaluated by their Rf value, TLC behavior, color, melting point, and IR spectral studies (Table 1).Qualitative analysis showed the presence of two important sterols i.e β-sitosterol (Rf- 0.60, mp 135-137^{o} C) and stigmasterol (Rf-0.55, mp 131-133 ^{o} C) (Table 1, Figure 1: A-B). A combination of other solvent systems such as benzene and ethyl acetate (85:15; Heble et al, 1968) and acetone and benzene (1:2; Khanna & Jain, 1973) were also used but solvent system of chloroform, hexane and acetone (23:5:2) was comparatively better than other solvent system.

Qualitative analysis of flavonoids showed the presence of three flavonoids viz., Kaempferol (Rf 0.81, brownish in visible light and blackish in UV after spraying 5% $FeCl_3$, yellowish in visible light and yellowish green in UV after spraying 5% $AlCl_3$, mp 271 ^{o}C -273 ^{o}C), Quercetin (Rf 0.75, brownish in visible light and blackish in UV after spraying 5% $FeCl_3$, yellowish in visible light and yellowish green in UV after spraying 5% $AlCl_3$, mp 309-311^{o}C) and Luteolin (Rf 0.78, brownish in visible light and blackish in UV after spraying 5% $FeCl_3$, yellowish in visible light and yellowish green in UV after spraying 5% $AlCl_3$, mp 326-329 ^{o}C) (Table 2, Figure 2;A-C).). They were confirmed on the basis of their Rf value, TLC behavior, color, melting point and IR studies (Table 2). The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of luteolin, quercetin and kaempferol in particular samples. Several others solvent systems such as n-butanol, acetic acid, water (4:1:5), tertiary butanol, acetic acid, water (3:1:1) were also tested, but the solvent system containing benzene, acetic acid, water (125:72:3) gave better results.

Quantitative Analysis

The phytochemicals which are present in different parts of *T. procumbens* were determined and quantified by standard procedures.

On quantification, the total amount of steroid content was found to be higher in unorganized mass i.e. callus as compared to stem and leaves. The total content of sterols in all the three tissues together is 5.88 mg/gdw and the order of sterol content in the three tissues is as follows

Callus (2.1±0.35 mg/gdw) > Stem (1.98mg±0.34 mg/gdw) > Leaves (1.8±0.23mg/gdw) (Table 3).

On Quantification, total flavonoids were found to be maximum in Callus, followed by stem and then leaves. The total flavonoid content in all three tissues together is 3.51 mg/gdw and the orede of flavonoid content in the three tissues is as follows:

Callus (1.38 mg/gdw) > Stem (1.11 mg/gdw) > Leaf (1.03 mg/gdw) (Table 4).

GC-MS Results

In the GC-MS analysis, 33 compounds were identified in the leaf and stem extracts of *T. procumbens*. The typical concentrations of the major compounds identified by GC-MS from leaf and stem extracts of *T. procumbens* are shown in Table 5, Table 6. In methanol leaf extract of *T. procumbens* highest peak (%) 14.65 was obtained by ethyl oleate, (RT 20.683) and the lowest peak area (%) 0.46 was obtained by 9-Octadecenamide (RT 23.119) (Table 5; Figure 3).

Whereas, in the benzene extract of stem the highest peak area (%) 14.24 was obtained by Tetracontane (RT 34.324) and the lowest peak area (%) 0.49 was obtained by 2, 8-Dimethyl-2- (4, 8, 12-Trimethyltridecyl)-6-CH (RT 31.208) (Table 6; Figure 4).The detailed tabulation of the GC-MS analysis of both the plant parts have been studied. The total ion chromatogram (TIC) showing the peak identities of the compounds have been identified using NIST library in the plant species.

The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula. The active principles with their Retention time (RT), Molecular formula, Molecular weight (MW) and peak area in percentage are presented in Table 5 &6 and Figure 3&4. The first compound identified with less retention time (5.08 min) was 1-Butanol, 3-methyl, format, whereas squalene was the last compound which took longest retention time (29.84min) to identify.

DISCUSSIONS

Natural products have been considered subjective to the effective maintenance of good health. It is estimated that about one third of currently marked drugs are related to natural products (Yu *et al.*, 2010). Analysis of various secondary metabolites by *in vivo* and *in vitro* experiments provides a reasonable approach towards understanding the biochemical basis of medicinal values of the plant species (Singh, 2004). Biosynthesis of secondary metabolites from intact plants, plant parts and tissue cultures has gained increasing attention over the years (Natesh, 2001; Mulabagal and Tsay 2004). Plants can synthesize different types of secondary metabolites, which were subsequently exploited by humans for their valuable role in a wide array of applications (Sam and Basu, 2008).

Secondary metabolites carry out a number of protective functions in the human body i.e they can boost the immune system, protect the body from free radicals, kill pathogenic germs etc (Chirdchupunseree and Pramyothin, 2010). A diet rich in plant food contains a range of secondary metabolites and contributes in protecting the body against cardiovascular illnesses and cancer. In recent years the use of secondary metabolites and their beneficial effects on humans are being intensively researched. They have wide array of applications like they act as defense machinery against pest and pathogens, providing protection against UV radiation and stress, or acting as attractive volatile odor compounds or pigments (Miyagawa, 2009).

Phytosterols are found in plant foods such as sunflower seeds, sesame, nuts and soyabeans. Phytosterols are known to protect against colon cancer and lower cholesterol levels. As the phytosterols are chemically identical to cholesterol, they compete against each other for absorption in the body and hence can lower the cholesterol levels. Presence of sterols has been reported from many plant species such as *Linum usitatissimum* (Herchi *et al*., 2009); *Chelonopsis albiflora* (Chen and Peng, 2009); *Goniothalamus laoticus* (Lekphrom *et al*., 2009); *Angelica polymorpha* (Li *et al*., 2009); *Phlomis umbrosa* (Liu *et al*., 2009); and *Acanthopanax sessiflorus* (Yang *et al*., 2009).

The free radical scavenging abilities of the structurally related steroids β-sitosterol , β-sitosterol glucoside (plant sterols and sterolins) show powerful skin properties by controlling lipid peroxidation (Backhouse *et al.,* 2008). Beta-sitosterol is mainly known and used for its cholesterol lowering property. Earlier reports on its phytochemical studies showed other health benefits like reducing risk of cancer, easing symptoms of benign prostatic enlargement, and by its antioxidant activity it prevents oxidative damage (Patra *et al.,* 2010).

Stigmasterol is another important constituent and has been isolated from plants. It is mainly involved in the synthesis of hormones like estrogens, progesterone, corticoids and androgens. In addition to stigmasterol many of its derivatives like, spinasterol, fucosterol, cyasterone, stigmasterol glucoside, fucosterol epoxide, stigma-4en-3one, 29-fluorostigmasterol etc. have been isolated and their pharmacological aspects have been assessed (Kaur *et al*., 2011). It was found by Chandler that stigmasterol has significant effect on serum cholesterol comparable with the antihypercholestrolemic activity of β-sitosterol. Stigmasterol, the active constituent of *Cacalia tangutica*, was found to be Cytotoxic to *Spodoptera litura* cells and its action was more marked in comparison to the other active constituents of the plant namely, friedelin and rotenone (Huang *et al*., 2009). *Carthami flos* contained stigmasterol which markedly inhibited the tumour promotion in the two-stage carcinogenesis experiments (Kasahara *et al*., 1994). Chloroform extract of *Parkia speciosa* was orally administered to the alloxan- induced diabetic rats and it was found to produce a significant depression in blood glucose levels (Jamaluddin *et al*., 1994). Stigmasterol present in bark of *Butea monosperma* showed decrease in hepatic lipid peroxidation and increase in the activities of catalase, superoxide dismutase and glutathione thereby suggesting its antioxidant property (Panda *et al*., 2009).

Thorns of *Gleditsia sinensis* was investigated for their active constituents and their antimutagenic activity (Jae-Chul *et al*., 2005). Acetone extract of *Sideritis foetens* was found to contain sterol fractions composed of stigmasterol, β-sitosterol and campesterol. These fractions were evaluated for their anti-inflammatory activity and they were found to reduce carrageenan induced paw oedema and also inhibited ear oedema induced by 12-O-tetradecanoylphorbol acetate (TPA) after topical application (Antonio *et al*., 2001). In the present study two valuable sterols such as β-sitosterol and stigma sterol were extracted and upon quantification showed a significant amount of these sterols ie. total sterols ranging from 1.8-2.1 mg/gdw were obtained from callus, leaf and stem tissues of *T. procumbens* (Table. 3). Earlier reports suggested the presence of β-sitosterol and stigmasterol imparts an anti-inflammatory response in *T. procumbens* (Gadre and Gadre, 1993). Similar results were also reported in another herb *Anoectochilus roxburghii* (Huang et al., 2007)

In vegetables and fruits naturally useful flavonoids like quercetin and kaempferol were ubiquitously present, and their antioxidant effect is highly helpful for human health (Wach *et al.,* 2007). Plant derived dietary polyphenols like quercetin and kaempferol have been extensively studied for their chemotherapeutic properties including antioxidant, anticancer and anti-inflammatory activites (Lamson and Brignale, 2000; Kumar *et al.,* 2014). It has been reported that these are “natural gifts” for the treatment of human cancer. Moreover better understanding their structure –activity relationships will pave way to synthesis analog compounds with improved stability, bio-availability, specificity and potency (Chen and Shi, 2009).

Our results in the present study showed significantly high amount of these important flavonoids like kaempferol ranged between 0.22-0.43 mg/gdw , quercetin ranged between 0.30-0.70 mg/gdw from leaf ,stem and callus cultures of *T.procembuns* (Table. 4). Earlier reports from *T. procumbens* extracted quercetin and kaempferol from callus cultures.

They showed effective antimicrobial activity against *staphylococcus aureus* and *candida albicans* (Jindal and Kumar, 2012). Shah et al (2012) using Reverse phase-HPLC also determined the presence of quercetin and kaempferol from methanolic extracts of *T.procumbens* whole plant. Quercetin and Kampferol also found to posses high antioxidant activity (Vellosa et al., 2011), anticancer activity (Vishnu priya et al, 2011), potential wound healing activity in *Ephorbia hirta* (Bigoniya et al, 2013).

Luteolin is another favorable nutrient from a class of naturally occurring molecules called bioflavonoids. Luteolin neutralizes free radicals such as the hydroxyl radical, superoxide, and other reactive oxygen species which prevent oxidative damage and may help reduce inflammation, regulate hyperactive immune systems and promote healthy carbohydrate metabolism (Harris *et al.*, 2006). In the present study useful luteolin was extracted from methanolic extracts of leaf, stem and callus cultures of *T. procumbens*, which upon quantification showed a significantly high amount ranging of 0.22-0.43 mg/gdw (Table 4). Presence of 5% Luteolin and glucoluteolin have been reported from *T. procumbens* flowers earlier (Verma and Gupta, 1988). Luteolin found to have anti-inflammatory, antinociceptive, anticarcinogenic, antimutagenic and antiangiogenic properties (Karrasch et al., 2007; Hendriks et al., 2004; Block et al, 1998). It shows strong antiproliferative activity against different human cancer cell lines as in solid malignancies and hormone dependent cancer lines e.g. breast, prostate, and thyroid cancer (Post and Varma, 1992; Ramanthan et al., 1993; Ryu et al, 1994). It is known from long that many higher plants are major sources of useful secondary metabolites which are used in agrochemical, pharmaceutical, aroma and flavor industries. Many useful plants are less explored, hence the search for new plant – derived chemicals should thus be a top priority in present and future efforts towards renewable conservation and rational utilization of biodiversity (Philipson, 1990). GC-MS is one of the best techniques to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids, esters etc. The GC-MS analysis of *C. italica* leaves revealed the presence of seventeen compounds (phytochemical constituents) that could contribute the medicinal quality of the plant. GC-MS analysis of methanolic extracts of the leaves and stem extracts of *T. procembuns* identified 33 compounds (Table 5, 6). The comparison of mass spectrum with NIST database library gave more than 90% identity as well as a confirmatory compound structure from literature. The obtained chemical acquaintance can be used for the further development of phytomedicines from the plant species. Furthermore, the presence of fatty acids in *T. procumbens* showed the pharmacological properties of the plant. GC-MS analysis clearly revealed the presence of saturated and unsaturated fatty acids, oleic acid, hexadecanoic acid, Octadecanoic acid, squalene etc. from the leaves and stem . This work will help in the future to identify the compounds, which may be used in body products, drugs, pharmaceutical and therapeutic values as well.

CONCLUSIONS

In summary, we conclude that in the present study significantly high amount of useful sterols like β-sistosterol and stigmasterol and flavonoids like quercetin, kaempferol and luteolin, were isolated and characterized both quantitatively and qualitatively. GC- MS analysis was performed and the results revealed the presence of bioactive compounds like ethyl oleate,9-Octadecenamide, Tetracontane, 2,8-Dimethyl-2-(4,8,12-trimethyltridecyl)-6-CH. Various saturated and unsaturated fatty acids, oleic acid, hexadecanoic acid, Octadecanoic acid, squalene etc were also detected from methanolic and benzene extracts of leaf and stem , respectively, tissues of this plant. Hence these results clearly indicate that this plant can be used for the further development of phytomedicines for therapeutic purposes.

ACKNOWLEDGEMENTS

First Author would like to thank head, Department of Botany, University of Rajasthan for support and providing necessary facilities to pursue research work.

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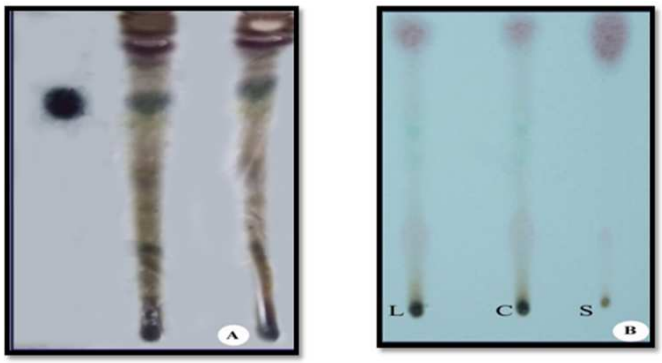
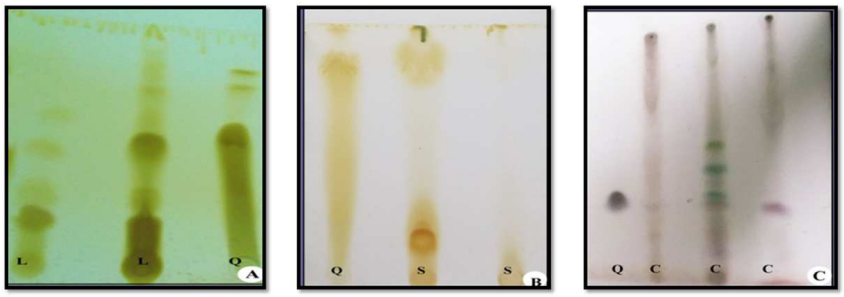
APPENDICES



A. Developed TLC of phytosterols in plant Stem with standard β-sitosterol
B. Developed TLC of phytosterols in plant parts: L-leaf; C-callus with S-standard (β-sitosterol

Figure 1: Developed TLC of Phytoserols



A. Developed TLC of Flavonoids in L- Leaf of *Tridax procumbens* with standard Q- Quercetin.
B Developed TLC of Flavonoids in S- Stem of *Tridax procumbens* with standard Q- Quercetin.
C. Developed TLC of Flavonoids in C- Callus of *Tridax procumbens* with standard Q- Quercetin.

Figure 2: Developed TLC of Flavonoids

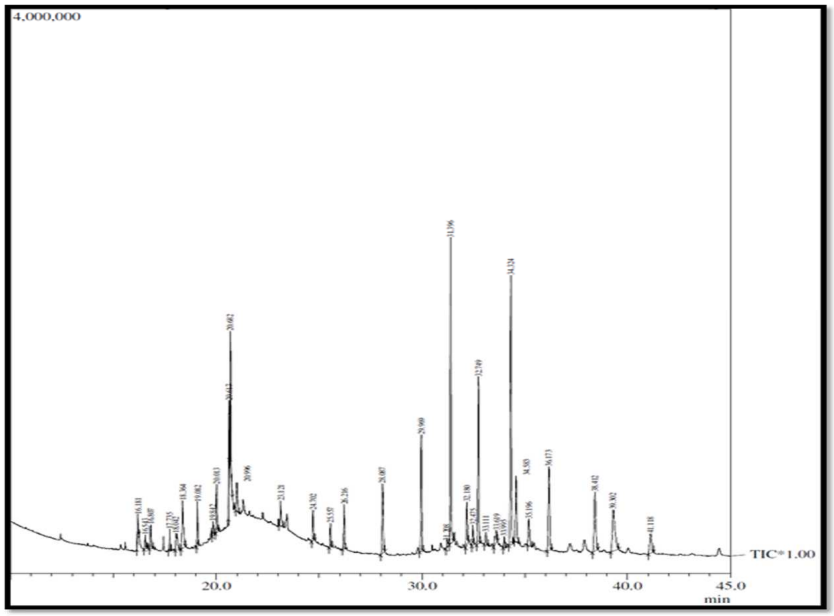


Figure 3: Chromatogram of Flavonoids in Leaf of *Tridax procumbens*

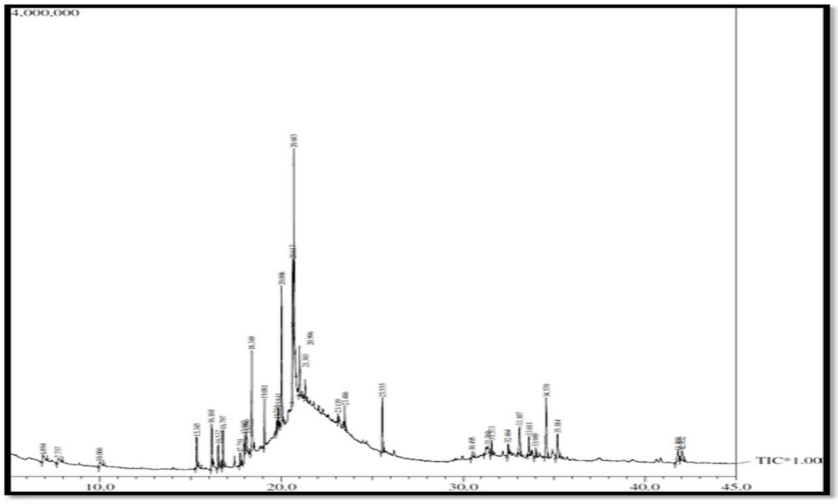


Figure 4: Chromatogram of Sterols in Stem of *Tridax Procumbens*

Table 1: Chromatographic Behavior and Physico-Chemical Characteristics of Isolated Phyto-Sterols Identified in *Tridax procumbens*

Isolated Compounds	R_f Value			Color After Spray or Physical Appearance		Melting Point (^oC)	IR Spectral Peaks ν (KBr) cm^{-1}
	S_1	S_2	S_3	R_1	R_2		
β-Sitosterol	0.89	0.90	0.71	PU-BN	PK	135-137	3350 (O-H), 2830, 1665 (C=C), 1470, 1300, 1052 (C-O) and 880
Stigmasterol	0.83	0.64	0.65	GY	PU	131-133	3400 (O-H). 2950, 1750, 1640 (C=O), 1035 (C-O), 991, 957, 935, 810 and 715

Abbreviations: *S_1 - Hexane : acetone (8 : 2), S_2 - Benzene : acetone (2 : 1), S_3 – Benzene : ethyl acetate (3 : 2), R_1 - 50% H_2SO_4, R_2 - Anisaldehyde reagent, BN - Brown, PK- Pink, PU – Purple, BL – Blue, GY – Gray.*

Table 2: Chromatographic Behavior and Physico-chemical Characteristics of Isolated Flavonoids identified in *Tridax procumbens*

Isolated Compounds	R_f Value			Physical Appearance			Color After SPRAY				Melting Point (°C)	IR Spectral Peaks ν (KBr) cm^{-1}
							R_1		R_2			
	S_1	S_2	S_3	Day Light	UV Ammonia	Iodine Vapor	Visible	UV	Visible	UV		
Kaempferol	0.86	0.83	0.55	GN-YW	BT-YW	YW-BN	BN	BK	YW	YW-GN	276-278	(O-H)-(3410cm-1(270, 295, 344, 1690
Luteolin	0.56	0.83	0.77	GN-YW	YW	YW-BN	TN	BK	DL-YW	YW-GN	326-329	3421, 2965, 1736 (lactone), 1510 (furan), 1461, 1388, 1360, 1274, 1242, 1187, 1136, 1028, 903, 850 cm^{-1}
Quercetin	0.78	0.64	0.41	GN-YW	YW	YW-BN	BT-GY	BK	DL-YW	YW-GN	315-320	3423, 1739, 1655 (O-H), 1608, 1508, 1305, 1203 (C=C), 1088.

Abbreviations: *S_1 – Benzene : acetic acid : water (125 : 72 : 3), S_2 – n-Butanol : acetic acid : water (4 : 1 : 5), S_3 – Conc. Hydrochloric acid : acetic acid : water (3 : 30 : 10), R_1 – 5% $FeCl_3$ solution, R_2 – 5% alc. $AlCl_3$ solution, YW – Yellow, BK – Black, BN – Brown, BT – Bright, DL – Dull, GN – Green, GY – Gray.*

Table 3: In Vivo and In vitro Sterols Content (mg/gdw) in *Tridax Procumbens*

Plant part	Leaves	Stem	Callus	Total
Sterols	1.8±0.23	1.98±0.34	2.1±0.35	5.88

Table 4: In Vivo and In Vitro Flavonoids Content (mg/gdw) in *Tridax procumbens*

	Free Flavonoids (mg/g. dw)				Bound Flavonoid (mg/g. dw)				Total Flavonoid (mg/g. dw)			
Plant Parts	Kaempferol	Luteolin	Quercetin	Total	Kaempferol	Luteolin	Quercetin	Total	Kaempferol	Luteolin	Quercetin	Total
Leaf	0.17	0.26	0.24	0.67	0.12	0.13	0.15	0.4	0.26	0.43	0.34	1.03
Callus	0.22	0.21	0.16	0.59	0.29	0.44	0.48	1.21	0.46	0.22	0.70	1.38
Stem	0.14	0.22	0.19	0.55	0.18	0.19	0.14	0.51	0.40	0.40	0.30	1.10
Total	0.53	0.71	0.59	1.81	0.59	0.76	0.77	2.12	1.12	1.05	1.34	3.51

Table 5: Phyto-Components (Flavonoids) Identified in the Methanol Extract of Leaf of *Tridax procumbens*

Peak	Retention Time	Area	Area %	Compound Name
1	6.894	404119	1.32	Hydroquinone
2	7.737	356794	1.1612	2-Methoxy-4-vinylphenol
3	10.006	286532	0.93	Benzene, 1-nitro-4-(phenylmethoxy)-
4	15.345	775441	2.53	2(4H)-Benzofuranone, 5,6,7,7A-Tetrahydro-6-H
5	16.168	792247	2.59	2,6,10-Trimethyl,14- ethylene-14-pentadecene
6	16.532	461471	1.51	3,7,11,15-Tetramethyl-2-hexadecene-1-ol
7	16.797	777965	2.54	3,7,11,15-Tetramethyl-2-hexadecene-1-ol
8	17.751	385394	1.26	
9	17.987	419426	1.37	n-Hexadecanoic acid
10	18.090	601449	1.96	Ethyl 9-Hexadecanoate
11	18.369	2794855	9.12	Hexadecanoicacid, Ethyl Ester
12	19.081	1014069	3.31	Hexadecanoicacid, trimethylsilyl ester

Table 5: Contd.,				
13	19.750	233545	0.76	9,12- Octadecadienoic acid (Z,Z)-
14	19.841	512175	1.67	9,12,15- Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
15	20.006	3373535	11.01	Phytol
16	20.617	1747256	5.70	Linoleic acid ethyl ester
17	20.683	4490548	14.65	Ethyl Oleate
18	20.996	1274433	4.16	Octadecanoic acid, ethyl ester
19	21.303	560374	1.83	Oleic acid, trimethylsilyl ester
20	23.119	142116	0.46	9- Octadecenamide
21	23.486	327082	1.07	Hexanedioic acid, bis(2-ethylexyl) phthalate
22	25.555	1444260	4.71	Bis (2-ethylexyl) ester
23	30.495	161392	0.53	Squalene
24	31.360	663257	2.16	Pentacyclol [19,3,1,1 (3,7), 1 (9,13), 1 (15,19)] octacosa- 1(25), 3, 5
25	31.573	355210	1.16	Cholesta-3,5-Diene
26	32.464	310708	1.01	Cholest- 5- ene, 3-Methoxy-, (3 Beta)-
27	33.107	1021401	3.33	Cholest-5-en—3-ol (3 beta)-, carbonochloridate
28	33.611	529836	1.73	Stigmasta-5,22-Dien-3-ol, acetat, (3-beta, 22-Z)
29	33.989	305185	1.00	Stigmast-5-en-3-ol, (3 Beta)-
30	34.570	2102968	6.86	Stigmast-5-en-3-ol, oleat
31	35.184	960706	3.13	Vitamin E
32	41.809	574814	1.88	Betulin
33	42.032	486335	1.59	4-(1,3,3-Trimethyl-bicycol[4,1,0] hept-2-yl)-but-3-en-2-one
		30646898	100.00	

Table 6: Phyto-Components (Sterols) Identified in the Methanol Extract of Stem of *Tridax procumbens*

Peak	Retention Time	Area	Area %	Compound Name
1	16.181	508206	1.02	2,610-Trimethyl,14-ethylene-14-pentadecene
2	16.543	301416	0.61	3,7,11,15-Tetramethyl-2-hexadecene-1-ol
3	16.807	5643721	1.13	3,7,11,15-Tetramethyl-2-hexadecene-1-ol
4	17.735	366107	0.74	Isophytol
5	18.042	719966	1.45	1,2-Benzenedicarboxylic acid,butyl 8-methylmonyl ester
6	18.364	1418923	2.85	Hexadecanoic acid,ethyl ester
7	19.082	799977	1.61	Hexadecanoic acid, trimethylsilyl ester
8	19.842	319334	0.64	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
9	20.013	1093933	2.20	2-Hexadecene-1-ol-3,7,11,15-tetramethyl-,[R-[R
10	20.617	1213806	2.44	Ethyl(9Z,12Z)-9,12-Octadecadienoate
11	20.682	2424837	4.87	Ethyl Oleate
12	20.996	741250	1.49	Octadecanoic acid, ethyl ester
13	23.121	653815	1.31	9-Octadecenamide
14	24.702	624584	1.26	Tetratetracontane
15	25.557	519132	1.04	Bis(2-ethylhexyl) phthalate
16	26.216	1019328	2.05	Hexacosane
17	28.087	1965169	3.95	Hexacosane
18	29.969	2667418	5.36	Hexatriacontane
19	31.208	246206	0.49	2,8-Dimethyl-2-(4,812-trimethyltridecyl)-6-CH
20	31.396	6319008	12.70	Tetracontane
21	32.180	1179994	2.37	2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-tri
22	32.475	453476	0.91	Cholest-5-ene,3-methoxyy-, (3 Beta)-
23	32.749	3840718	7.72	Hexatriacontane
24	33.111	409529	0.82	Cholest-5-ene,3-ol (3-beta)-, carbonchloridate
25	33.619	534549	1.07	Stigmasta-5,22-dien-3-ol, acetat, (3-beta,22-Z)
26	33.995	308033	0.62	Stigmast-5-en-3-ol, (3Beta)-
27	34.324	7087401	14.24	Tetracontane
28	34.583	1923473	3.87	Stigmast-5-en-3-ol,oleat
29	35.196	906770	1.82	Vitamin E
30	36.173	2801878	5.63	Tetracontane
31	38.412	2354300	4.73	Dotriacontane
32	39.302	2588237	5.20	Stigmast-5-en-3-ol, 3 (Beta)-
33	41.118	890740	1.79	Hexatriacontane
		49765885	100.00	