Research Article

Structural Characterization and Evaluation of Antioxidant Activity of Isolated Phenolic Compounds from *Talinum fruticosum* Leaves

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ABSTRACT

The present investigation appraises the antioxidant properties, total phenolic content and isolation and characterization of bioactive principles from *Talinum fruticosum,* a leafy vegetable. The herbal extracts of the leaves were prepared with water, ethanol, methanol, ethyl acetate and hexane at 10 % concentration levels. Radical attenuating abilities of the 5 plant extracts were ascertained by 2, 2-diphenyl 1-picryl hydrazyl, superoxide, hydroxyl radical scavenging and Ferric reducing ability of Plasma assays. Methanolic extract showed highest antioxidant activity with a direct relationship between activity and polyphenolic content. HPLC profile of silica gel column purified fractions – 1 and 2 revealed single major peaks with retention times 2.7 and 2.5min respectively. The ^{1}H and ^{13}C NMR, FTIR spectroscopic analysis of the purified fractions – 1 and 2 suggest the compounds to be Kaempferol-7-O-neohesperidoside and Luteolin. Characterization of phenolics compounds substantiates its stake as leafy vegetable rich in antioxidants.

Keywords: *Talinum fruticosum*, Antioxidant activity, Total phenolic content, HPLC, FTIR, NMR.

INTRODUCTION

Oxygen is necessary for the release of energy and sustenance of life. Normally oxygen is reduced to water. Incomplete oxidation of oxygen gives rise to various reactive molecules called reactive oxygen species (ROS). These include superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) nitric monoxide ($NO^{\bullet}$), peroxynitrile and hypochlorous acid. These oxygen derived free radicals give rise to cell injury through DNA and protein modification and lipid peroxidation. The resultant cellular damage produces different pathologies in human body.

There should be a balance between production of ROS and protection from ROS. Any imbalance between these leads to a condition called oxidative stress[1]. Free radicals are produced due to excessive production of ROS, loss of antioxidants causing damage to nucleic acid bases, lipids, proteins affecting cell health, viability and induce cellular responses. Oxygen generated free radicals eventually lead to cell death by necrosis or apoptosis[2] and are also either directly or indirectly associated with clinical and chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging, pulmonary toxicity, macular degeneration, cataract and neurodegenerative diseases in humans[3].

Free radical formation is controlled naturally by several useful compounds known as antioxidants. Antioxidants protect the body from damaging oxidation reactions by reacting with free radicals. Antioxidants are proficient in stabilizing or deactivating free radicals before they attack cells[4]. Epidemiological studies reported that many of antioxidant compounds owe anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial and anti-viral activities in varied magnitude. In many cases, increased oxidative stress is associated in the development and progression of diseases by increased generation of free radicals or failure of antioxidant defense. However, the studies testify that intake of natural antioxidants reduce risk of cancer, cardiovascular diseases, diabetes and other diseases associated with aging, etc. Although there are some manmade antioxidant compounds such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA), propyl gallate (PG) and tertiary butylhydro quinone (TBHQ), etc. that are usually used in processed foods to prevent deterioration, nutritional losses and off flavouring, etc. Previous literature reports put in the picture that these manmade compounds have some side effects. Alternatively there are some natural antioxidants which are useful to mankind, safe and also eco-friendly. These are the naturally occurring phytochemicals or phytonutrients found in abundance in Nature[5].

Phytochemicals or phytonutrients from medicinal plants are plant products, plant foods, such as fruits, leaves, seeds etc. which are easily available sources of antioxidants[6]. Since time immemorial medicinal plants played a vital role in the cure and prevention of diseases. Researchers proposed that a diet rich in antioxidants from fruits, vegetables and leafy vegetables are associated with a lower risk of life threatening diseases and plant-based diet protects against oxidative stress-related diseases[7].

Bioactive compounds extracted from plants were found to be effective in food systems and are employed as additives in food industry as antioxidants and

antimicrobials. Use of synthetic antioxidants in cosmetic and pharmaceutical industry is dissuaded due to their known carcinogenic effects. Discovery and utilization of traditional medicinal food plants serves as a valuable alternative to synthetic antioxidants[8].

Talinum fruticosum, commonly known as Ceylon spinach is used as a leafy vegetable. Various other species of *Talinum* are found to be rich in antioxidants, phytochemicals and phytonutrients. In some parts of India especially Assam it is used to treat diabetes. Previous studies show that, crude leaf extracts of *Talinum triangulare* another species of genus *Talinum* is of therapeutic value in treating cardiovascular diseases, stroke, obesity, polyuria, internal heat, measles, gastrointestinal disorders, hepatic ailments and cancer[9]. Further the active principles are to be identified and characterized for pharmaceutical and therapeutic applications.

In the present study, leaves of *Talinum fruticosum* were investigated for their phytochemical constituents, antioxidant activity and total phenolic content. The active principles identified are further purified and characterized to endorse the nutritive and medicinal value of this plant.

MATERIALS AND METHODS

Preparation of plant extract

Leaves of *Talinum fruticosum* are collected from a vegetable garden in Vizianagaram, Andhra Pradesh, India. The plant is authenticated by Dr. K. Manikya Kumari, Department of Botany, St. Joseph's college for Women, Visakhapatnam.

Leaves were weighed to 10gms, washed and shade dried. The leaves were homogenized using motor and pestle and the ground leaves are soaked separately in 100ml each of n-hexane, methanol, ethyl acetate, ethanol and distilled water for 10days. The extracts were filtered through muslin cloth to remove the debris. The extracts were vacuum dried using a rotary evaporator. The dried powder is dissolved in appropriate solvents and is used for further analysis.

Phytochemical analysis

Phytochemical analysis is a preliminary screening test to identify the secondary metabolites. In the present study, several phytochemical constituents were evaluated qualitatively using standard protocols[10,11].

Test for Alkaloids

Mayer's test: To 1ml of plant extract few drops of Mayer's reagent was added along the walls of the test tube. A creamy colored precipitate indicates the presence of alkaloids.

Tests for Polyphenols

Wiefferering test: 1ml of 2% copper sulphate solution and few drops of conc. HCl were added to 1ml of plant extracts. Formation of reddish-violet precipitate indicates the presence of polyphenols.

Test for Flavonoids

To 1ml of plant extract, 1ml of 10% NaOH was added. Increased intensity of yellow color which disappears on addition of few drops of conc. HCl, indicates the presence of flavonoids.

Test for Terpenoids

To 1ml of plant extract, 2ml of chloroform and 2ml of H_2SO_4were added. Formation of reddish brown color indicates the presence of terpenoids.

Test for Saponins

Foam and froth test:1 ml of plant extract was treated with 2ml of distilled water. The suspension was shaken for 15 min. A two cm layer of foam which is stable for 10 min indicates the presence of saponins.

Test for Tannins

Ferric chloride test: To 1ml of plant extracts 2ml of 10% $FeCl_3$ was added, formation of green color indicates the presence of tannins.

Test for Glycosides

To 1ml of plant extract 3ml of chloroform and 3ml of ammonia solution were added. Formation of pink color indicates the presence of glycosides.

Test for steroids

To 1ml of plant extract 2ml of chloroform and 2ml of conc. H_2SO_4 were added, formation of yellowish green fluorescence indicates the presence of steroids.

Test for Quinones

To 1ml of plant extract, 1ml of conc. H_2SO_4 was added. Formation of red color indicates the presence of quinones.

Test for Coumarins

To 1ml of plant extract, 1ml of 10% NaOH was added; formation of intense red color indicates the presence of coumarins.

Total phenolic content

Total phenolic content was determined by modified method of Siddique[12,13] using Folin Cio-calteau reagent. To 100µg of plant extract, 0.5 ml of distilled water, 2.0ml Folin Cio-calteau reagent and 2.0ml 7.5% Na $_2CO_3$ were added. The mixture was allowed to stand for 15 min at 45^{o}C and the absorbance was measured at 765 nm using spectrophotometer. The standard calibration curve was prepared using Gallic acid in the range of 10-50µg. Total phenolics levels were expressed as Gallic acid equivalents (mg g^{-1} of dry mass).

Antioxidant activity

DPPH radical scavenging assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined by the method of Wong[14]. To 5 ml of methanolic DPPH, 50µl of plant extract was added. The reaction mixture was incubated at 37°C for 30 min in dark and the absorbance was read at 517nm using spectrophotometer. BHT was used as standard and for control; plant extract was replaced by appropriate solvent. The percent of inhibition was calculated using the equation

% I = Ab of C – Ab of S/ Ab of C X 100

% I = % of inhibition

Ab of C = absorbance of control

Ab of S = absorbance of the plant extract.

Superoxide radical scavenging activity

Superoxide dismutase (SOD) is a metalloenzyme that catalyzes the superoxide radical into hydrogen peroxide (H_2O_2) and oxygen (O_2) and subsequently play a significant defense role against superoxide radical toxicity. SOD activity was determined by the method of Beauchamp and Fridovich[15] with some revisions. The superoxide anions were generated in a reaction mixture containing 1.0ml of sodium carbonate (125mM), 0.4ml NBT (24µM) and 0.2 ml of EDTA (0.1mM). The reaction was initiated by addition of 0.4ml of hydroxylamine hydrochloride (1mM) and 0.5ml of different plant extracts. Reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560nm. In control, plant extract was replaced by appropriate solvent. BHT (1mg/ml) was used as a standard. The percent of inhibition of superoxide radical was calculated as

% I = Ab of C – Ab of S/Ab of C X 100

Hydroxyl radical scavenging activity

Hydroxyl radical is one of the effective oxygen species in the biological system. It reacts with cell membrane phospholipids and causes damage to cell. It was assayed by Kunchandy and Rao method[16] with some adaptations. The reaction mixture contains 100µl of plant extract, 500µl of each (0.6mM) of deoxyribose in phosphate buffer (20mM, pH 7.4), ferric chloride (0.1mM), EDTA(0.1mM), and ascorbic acid (0.1mM), 100 µl of H_2O_2 (1mM) and 800µl of phosphate buffer in a final volume of 3ml. After incubation at 37°C for 1hr, 1.0 ml of each of TCA (2.8%) and thiobarbituric acid (TBA) (1%) were added and kept in water bath at 100 °C for 20 min. The mixture was allowed to cool at room temperature, centrifuged at 4000rpm for 15 min. The absorbance of the supernatant was read at 532 nm. BHT (1mg/ml) was used as standard, for control plant extract was replaced by suitable solvent. The percent of inhibition was calculated from following equation

% I = Ab of C – Ab of S/ Ab of C X 100

Total antioxidant activity by FRAP method

Ferric chloride reducing ability of plasma was used to define the total antioxidant capacity of plant extracts as designed by the method of Benzie and Strain[17]. In this assay, the ability of reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) to ferrous tripyridyltriazine (Fe(II)-TPTZ) by plant extracts was considered as total antioxidant activity. In the presence of antioxidants, Fe^{+3}–TPTZ complex is reduced to Fe^{+2}–TPTZ complex which gives an intense blue color with maximum absorbance at 517nm. A calibration curve was drawn using $FeSO_4$ with concentration ranging from 1 – 10mM. To 1.5ml of FRAP reagent, 50µl of plant extract was added and the absorbance was read at 517nm and results are expressed as Ascorbic acid Equivalent Antioxidant Capacity (AEAC) in terms of µM.

Purification

The extracts which are having high antioxidant activity are purified and further characterized.

Column chromatography

The methanol extract was purified using silica gel with mesh size of 100–300µm on a column with dimension of 10x300mm. It was packed with activated silica gel using n-hexane and washed with 2 bed volumes of same solvent. Methanolic leaf extract was reconstituted with same solvent and loaded onto packed silica gel column. The fractions were eluted with ethyl acetate: methanol combination in the ratio of 3:1, 2:2, 1:3 and 4. Fractions of 3ml each were collected and their absorbance was measured over a range of 190 to 800nm. Similar fractions were pooled and analyzed by thin layer chromatography (TLC) for homogeneity[18].

Thin layer chromatography (TLC)

The purified fractions of methanol extract were subjected to TLC (0.5 mm, 20×20 cm), performed on glass plates coated with adsorbent silica gel G 60 and solvent system constituting ethyl acetate: formic acid: water (8:1:1). 15ml of 3% of Boric acid and 5ml of 10% oxalic acid was used as developing reagent. The sprayed plates are incubated at 80°C for 15mins and examined under UV trans illuminator[19].

High Performance Liquid Chromatography (HPLC)

Purity of the compounds was analyzed by HPLC. With some modifications of Adithya[20] the eluant fractions from silica gel column were further separated by reverse-phase HPLC, on a C_{18} column (Shimadzu) that was previously equilibrated with the mobile phase containing acetonitrile: water in 2:3 ratio. HPLC was performed with a flow rate of 1.0 ml/min for 30 min employing binary gradient elution. The peaks were monitored at 290 nm using UV detector.

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR spectroscopy was used to analyze and identify functional groups as designated by Kumar[21]. The solid state FTIR spectra (4000 - 400 cm^{-1}) are recorded on Perkin Elmer. The sample was prepared by grinding the purified sample with potassium bromide (KBr) to get pellet.

Nuclear Magnetic Resonance spectroscopy (NMR)

Purified antioxidant compounds were further characterized by NMR Spectroscopy. The ^{1}Hand ^{13}C NMR spectra were recorded on a BRUKERAv 400, Andhra University, India, in DMSO- D_6 solvent using TMS as reference[22].

RESULTS AND DISCUSSION

The results demonstrated that poly phenols, flavonoids and coumarins were ubiquitous in all the five leaf extracts of *T. fruticosum* on preliminary phytochemical analysis.

Alkaloids, steroids, saponins and glycosides were completely absent in all the 5 extracts Tannins were present in all the extracts except hexane and terpenoids were absent in aqueous and ethyl acetate extracts. Quinones are present in all the leaf extracts except in ethanol extract (Table – 1).

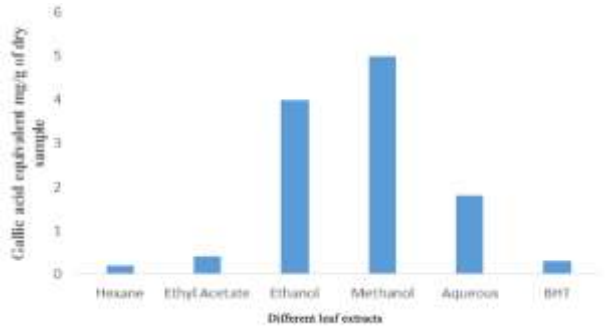
Although tannins are present in all the polar solvents, they can be removed by cooking as they become solubilized. Absence of alkaloids and saponins further substantiates the plant as a preferred vegetable source.

Figure – 1 reveals the total phenolic content in all the leaf extracts of *T. fruticosum.* The total phenolic content ranges from 0.2 to 5mg of Gallic acid/g dry sample.

The highest content was found in methanol extract of *T. fruticosum* with 5mg g^{-1} followed by ethanol, aqueous, ethyl acetate extracts and the lowest in hexane extract with 0.2mg g^{-1}.

The outcome of the total phenolic content supports the hypothesis that phenol rich plants are good sources of natural antioxidants[12,13,23].

Presence of phenolic compounds in most of the plants envisages antioxidant activity. Phenolics with hydroxy groups in methanolic extracts of various plants scavenge free radicals and ROS[24].



Legend: Total phenolic content calculated as GAE in mg g^{-1} dry weight.

Figure 1: Total phenolic content of different extracts of *T. fruticosum*

Various methods are used to investigate the antioxidant property of samples In the present study, DPPH, Superoxide radical, H_2O_2 scavenging activities and FRAP methods were used to assess total antioxidant capacity of the different solvent extracts and the results are shown in figure-2 and tables-2. Substantial differences were observed among the antioxidant activities of the 5 solvent extracts assessed. The methanolic extract obtained a high total antioxidant capacity with DPPH, superoxide radical scavenging activity and FRAP, except for hydroxyl radical scavenging activity, which was best in aqueous extract.

DPPH free radical is a stable radical giving violet color in methanol and decolorizes on reduction by donating hydrogen or electron in the presence of an antioxidant[25]. Methanol extract demonstrated highest inhibition with 91.31% followed by other solvents, ethanol (85.05%), ethyl acetate (73.14%), aqueous (50.33%) and hexane extracts (48.11%). DPPH radical scavenging activity was compared with BHT (1mg/ml), which has displayed 60.38%inhibition (Table–2).

Table 1: Phytochemical analysis of leaf extracts of *T. fruticosum*

Various Tests	Hexane extract	Ethyl acetate extract	Ethanol extract	Methanol extract	Aqueous extract
Alkaloids	-ve	-ve	-ve	-ve	-ve
Polyphenols	+ve	+ve	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve	+ve
Terpenoids	+ve	-ve	+ve	+ve	-ve
Saponins	-ve	-ve	-ve	-ve	-ve
Tanins	-ve	+ve	+ve	+ve	+ve
Glycosides	-ve	-ve	-ve	-ve	-ve
Steroids	-ve	-ve	-ve	-ve	-ve
Quinones	+ve	+ve	-ve	+ve	+ve
Coumarins	+ve	+ve	+ve	+ve	+ve

Legend: -ve = phytochemicals absent; +ve = phytochemicals present

Table 2: Antioxidant activities of *T. fruticosum* leaf extracts

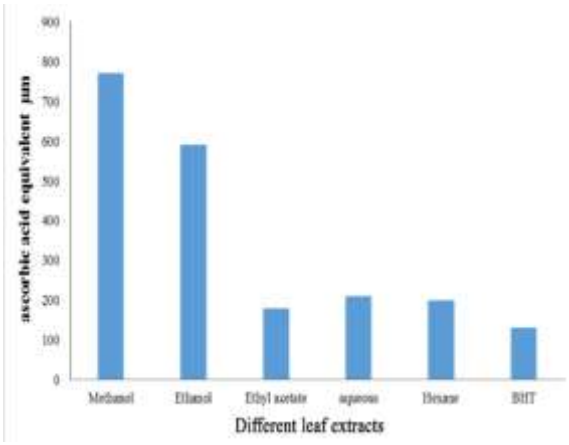
Various antioxidant scavenging assays	Different solvent extracts					
	Methanol	Ethanol	Ethyl acetate	Aqueous	Hexane	BHT
DPPH radical scavenging	91.31%	85.05%	73.14%	50.33%	48.11%	60.38%
Superoxide radical scavenging	99.17%	95.79%	80.34%	82.26%	81.20%	89.86%
Hydroxyl radical scavenging	73.48%	35.29%	57.79%	79.89%	77.24%	60.06%

Legend: Percent Inhibition of DPPH, superoxide and hydroxyl radical scavenging activities

Superoxide radical is strongest reactive oxygen species among free radicals and produced from molecular oxygen by both enzymatic and non-enzymatic reactions[26]. Superoxide radicals convert harmful oxygen species such as hydrogen peroxide by the action of dismutase, which further converted to reactive hydroxyl radicals in the presence of certain metal ions. Hydroxyl radicals attack DNA and causes strand necrosis, leading to harmful diseases. Superoxide radical scavenging activity of plant extracts was checked by the reduction of Nitroblue Tetrazolium which gives rise to NBT diformazon. The rate of reaction with oxygen is related to xanthine oxidase activity and inhibited by SOD[27]. Percent superoxide radical scavenging activity of different extracts of *T. fruticosum* in the present study was given in table–2. Methanol extract exhibited prominent and highest activity compared to standard BHT and other solvent extracts. The percent inhibition is in the order: methanol extract-99.17%, ethanol – 95.79%, aqueous–82.26%, hexane-81.20% and the lowest is observed in ethylacetate extract–80.34%.

When hydrogen peroxide is converted into hydroxyl radicals, they cause toxicity to living cells by reacting with biomolecules causing cell damage and death[28]. It is one of the strongest reactive oxygen species and radical induced pathogenesis is due to hydroxyl radicals. This is a totally aqueous system in which ascorbic acid, iron and EDTA combine with each other to generate hydroxyl radicals[27]. Effect of hydroxyl radical is evidenced by the degradation of 2-deoxyribose giving pink color in the presence of TBA. Results of the present study (Table-2) revealed that aqueous extract achieved better results compared to other extracts. Among the 5 extracts under study ethanol extract showed a lowest antioxidant activity.

The FRAP assay is a simple, inexpensive and highly reproducible method, based totally on electron transfer and is very useful to distinguish dominant mechanisms with antioxidants[17,29]. Reduction of ferric to ferrous ion at low pH gives the formation of colored ferrous-tripyridyltriazine complex. Antioxidant activity is a measure of change in absorbance by solvent extracts compared to that of ascorbic acid at 517nm (Ascorbic Acid Equivalents, AEAs). Figure–2 show that the highly nonpolarhexane extract exhibited lowest reducing power. The methanol extract showed a prominent activity compared with other extracts and standard synthetic antioxidant BHT. The total antioxidant capacity was determined and expressed in µM of Ascorbic acid equivalent.



Legend: Ascorbic acid content in different leaf extracts.

Figure 2: FRAP assay in different leaf extracts of *T. fruticosum*

Antioxidant activity of plant extracts not only depend on extract composition but also on the methods of determination. Various methods measuring the total antioxidant activity *in vitro* can be classified into 2 types: Assays based on hydrogen atom transfer (HAT) and Assays based on electron transfer (ET). HAT-based assays are the ones in which antioxidant and substrate compete for thermally generate peroxyl radical. ET based assays measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentration. Total phenols assay by Folin Cio-calteu reagent, DPPH, SOD and FRAP assay are ET based assays[30]. Each method exhibits a different mode of action hence more than one type of antioxidant capacity measurements needs to be performed.

Super oxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals[31]. Further, Superoxide radical is considered to play an important role in the peroxidation of lipids[32]. The superoxide radical activity supersedes both DPPH and hydroxyl radicals scavenging activities. Methanol extract has shown better results compared to other solvent extracts, except for hydroxyl radical scavenging activity table–2 and also methanol extract is chosen as the best solvent, to extract antioxidant compounds from *T.*

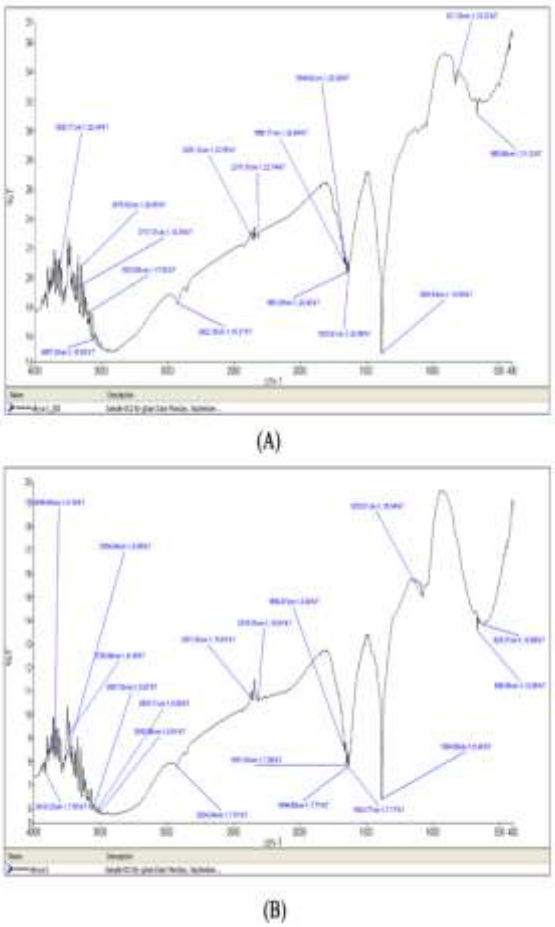
fruticosum. Natural antioxidants are multifunctional and are alternative to synthetic antioxidants in food. Their activity depends on the several factors such as the multiplicity and heterogeneity of the plant and the investigation conditions used for the product[23].

Taking into account the outstanding antioxidant potential and polyphenolic content of methanolic extract of *T. fruticosum* it is further purified by column chromatography. Organic solvents run fast in silica column owing to stronger affinity of silica gel to polar components, the low polar molecules will elute first[18]. Methanol extract (0.5g) is loaded on silica column and ethyl acetate and methanol gradient was used as mobile phase in different ratios (3:1, 2:2, 1:3 & 4). 3ml fractions were collected and the absorbance measured at 190-720nm. The spectrum of each fraction is analyzed; fractions exhibiting similar spectra are pooled. Fractions 6 to 9 formed the purified fraction - 1 and 11 to 14 purified fraction – 2. The purified fractions are evaporated to dryness and weighed. Purified fraction – 1 has given 21mg/10g weight of leaf sample and purified fraction – 2 gave 19mg/10g weight of leaf sample that are used for further analysis.

A volume of 1µl each of 1% purified fraction – 1 and 2 when spotted on TLC plates gave fluorescent yellowish green colored spots. The one dimensional TLC a revealed the presence of flavonoid group of compounds and a single band indicates that the fractions are pure. Flavonoids treated with boric and oxalic acids give compounds which after heating fluorescence yellowish green. The phytochemical analysis and TLC incline towards the flavanoid nature of the purified fractions.

The HPLC chromatographic patterns of the methanolic extracts of leaf samples for Purified fraction–1 and 2 monitored at 290nms. Single prominent peaks were detected at 290nms having t_R of 2.7 and 2.5min respectively. The TLC and HPLC results are in congruence with one and other with respect to the purity of the sample.

The FTIR studies reveal the functional groups present in the purified samples. Purified fraction-1 FTIR spectrum exhibited peaks at ~ 3400 (broad), 2922and 2890cm^{-1}attributed to OH group, asymmetric CH_3 stretching and symmetric CH_3 stretching. The peaks at 1660 and 1651cm^{-1} are assigned to carbonyl group however, 1633 and 1304cm^{-1} denotes the presence of aromatic ring. The peak observed at 1030cm^{-1}could be attributed to –C–O–C. The observed peaks in the purified fraction-2are 3515 and 2924cm^{-1} assigned to OH group and CH_3 stretching respectively. Peaks at 1651 and 1644cm^{-1} denotes the presence of C=O of carbonyl group and 1633cm^{-1} represents the C = C of aromatic ring, 1078cm^{-1} denotes the –C–O–C. IR spectrum of purified fraction 1 and 2 revealed the presence of OH, carbonyl group and aromatic ring as well as CH_3 groups (Figure – 3A & 3B).



Legend - 3A & B: FTIR spectrum of purified fraction 1 and 2 ranging from 4000 – 600cm^{-1}

Figure 3A & B: FTIR Spectrum of Purified fraction – 1 and 2

The structure of purified fraction 1 and 2 has been determined by both ^{1}H NMR and ^{13}C NMR. The^{1}H NMR analysis for purified fraction-1at δ 7.32, 7.080, 7.89 and 7.87 are indicating the presence of aromaticring and δ 8.34, 8.128 and 8.109 are indicating the hydroxyl groups (-OH). However, the peaks at 5.77 – 5.31 for CH protons and at 4.8 – 4.5 for CH_2 proton.

In the ^{13}C NMR a small peak was observed at 200ppm that can be assigned to C = O. Few small peaks were also noticed at 135, 130, 128 and 100ppm for aromatic protons. The peaks at 71.54, 70.69, 69.26, 68.89, 61.08 and 54.89 are assigned to CH_2 carbons of ring A and B. The peaks observed at 29.46 and 25.66ppm are due to CH_3 carbon (Figure – 4A & 5A). For purified fraction-2, δ 7.87 – 7.85 representing aromatic protons, δ 1.2 indicates CH_3 and 2.0 indicates CH_2 protons. In the ^{13}C NMR a small peak was observed at 207 and 203ppm can be assigned to C = O. The peaks were also noticed at 141.77 and 138.50ppm for aromatic protons. The peaks at 89.35, 84.12, 73.77and 72.33ppm can be assigned to various carbons attached with OH (Figure – 4B & 5B).

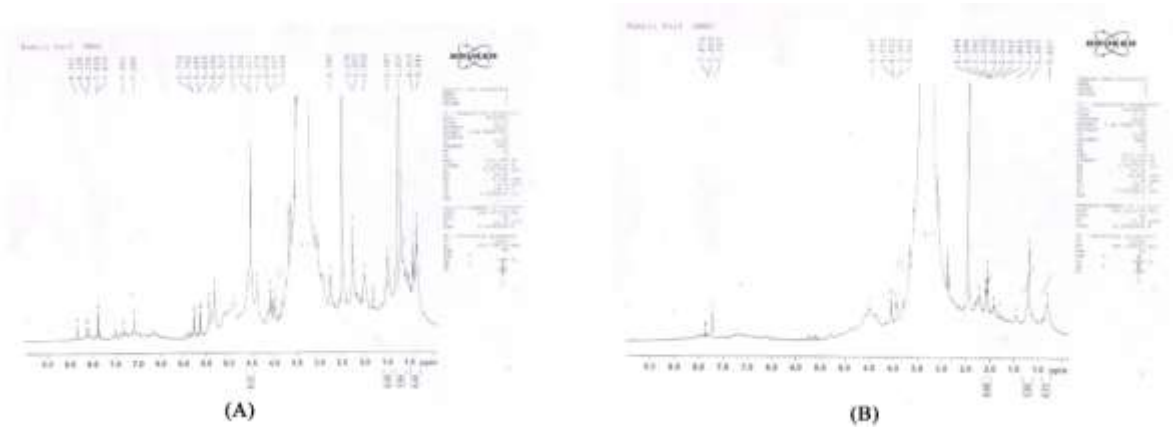


Figure 4A & B: The ^{1}H NMR Spectrum of Purified fraction – 1 and 2

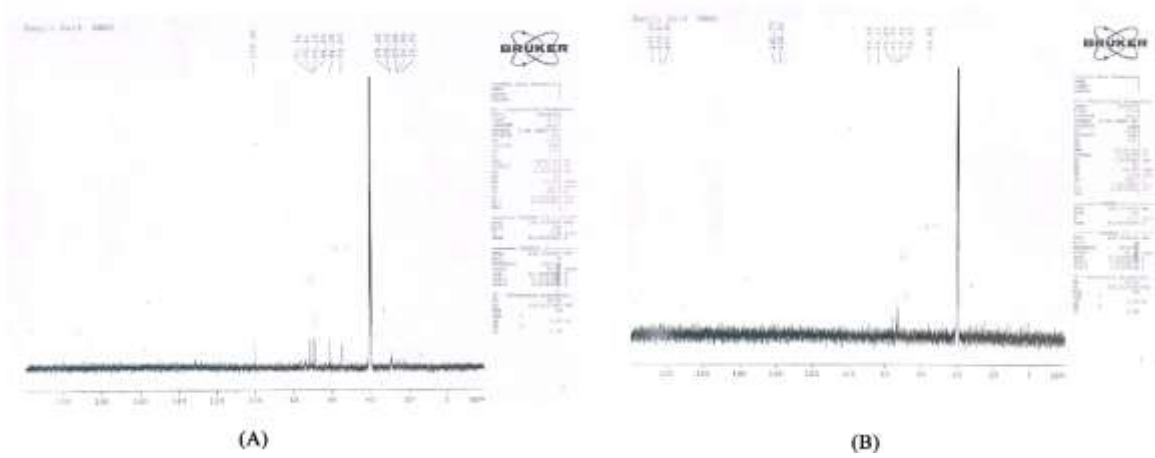
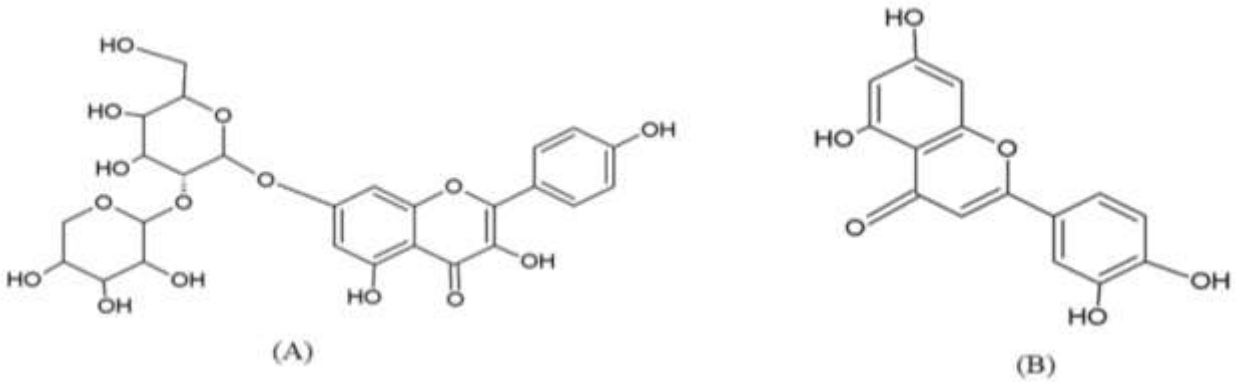


Figure 5A & B: The ^{13}C NMR Spectrum of purified fraction – 1 and 2

On the basis of above FTIR and NMR results the purified fraction-1 may be a Kaempferol-7-O-neohesperidoside and purified fraction-2 may be a Luteolin (Figure – 5A & 5B). Further structural analysis is under process in our laboratory.



Legend - 6A &8B: A Kaempferol-7-O-neohesperidoside and A Luteoline

Figure 6A & B: Proposed structure of Purified fraction – 1 and 2

CONCLUSION

In the present study, a comprehensive analysis of the phytochemical constituents and antioxidant capacity of the *T. fruticosum* was carried out. The results demonstrate that *T. fruticosum* leaves are a rich source of phenolic compounds. The methanol extract of *T. fruticosum* has shown highest antioxidant activity *in vitro* against various radicals as against the synthetic antioxidant BHT. Methanol extract on further purification yielded 2 bioactive molecules that are characterized as Kaempferol-7-O-neohesperidoside and Luteolin. These results substantiate the presence of flavones and flavonoids (phenolic compounds) there by, *T. fruticosum* can serve as a valuable resource in pharmaceutical and food industry.

From this study *T. fruticosum* is authenticated as a natural source of antioxidant in the form of a leafy vegetable.

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REFERENCES

1. Piconi L., Quagliaro L., and Ceriello A. Oxidative stress in diabetes. *Clin. Chem. Lab. Med.* 41, 2003, 1144–1149.
2. Dalle-Donne I., Rossi R., Colombo R., Giustarini D., and Milzani A. (2006) Biomarkers of oxidative damage in human disease. *Clin. Chem.* 52, 2006, 601–623.
3. Uttara B., Singh A. V, Zamboni P., and Mahajan R. T. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* 7, 2009, 65–74.
4. Percival, M. Antioxidants. *Clin. Nutr. insights,* 1998, 1–4.
5. Rao T. N., Kumarappan C., and Lakshmi S. M. Antioxidant Activity of Talinum portulacifolium (Forssk) Leaf Extracts, 8, 2008, 329–338.
6. Nanjo F., Goto K., Seto R., Suzuki M., Sakai M., and Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radic. Biol. Med.* 21, 1996, 895–902.
7. Sharma S. K., Singh L., and Singh S. a Review on Medicinal Plants Having Antioxidant Potential, 1, 2013, 404–409.
8. Pu W., Wang D., and Zhou D. Structural Characterization and Evaluation of the Antioxidant Activity of Phenolic Compounds from Astragalus taipaishanensis and Their Structure-Activity Relationship. *Sci. Rep.* 5, 2015, 13914.
9. Rao N., Mittal S., and Menghani E. (2013) Academic Sciences 5.
10. Agnel Arul John N., and Shobana G. Anti-inflammatory activity of Talinum fruticosum L. on formalin induced paw edema in albino rats. *J. Appl. Pharm. Sci.* 2, 2012, 123–127.
11. Firdouse S., and Alam P. Short communication 3, 2011, 32–35.
12. Siddique N. A., Mujeeb M., Najmi A. K., Khan H. N., and Farooqi H. WITHDRAWN: Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of Aegle marmelos. *J. Saudi Chem. Soc.* 4, 2010, 1–5.
13. Soares M. O., Alves R. C., Pires P. C., Oliveira M. B. P. P. and Vinha A. F. Angolan Cymbopogon citratus used for therapeutic benefits: Nutritional composition and influence of solvents in phytochemicals content and antioxidant activity of leaf extracts. *Food Chem. Toxicol.* 60, 2013, 413–418.
14. Wong C. C., Li H. Bin, Cheng K. W., and Chen F. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chem.* 97, 2006, 705–711.
15. Beauchamp C., and Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44, 1971, 276–287.
16. Kunchandy E., and Rao M. N. A. (1990) Oxygen radical scavenging activity of curcumin. *Int. J. Pharm.* 58, 1990, 237–240.
17. Benzie I. F., and Strain J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* 239, 1996, 70–76.
18. Wilson K., and Walker J. Principles and Techniques of Biochemistry and Molecular Biology. *Director*.
19. El-Olemy M., Al-Muhtadi F., and Afifi a. Thin Layer Chromatography Characterization of Flavonoids 1–5.
20. Es A., Jm S., and Ka K. (2012) Academic Sciences 4.
21. Kumar A., Vara N., and Majeti P. Proteomic responses to lead-induced oxidative stress in Talinum triangulare Jacq. (Willd.) roots: identification of key biomarkers related to glutathione metabolisms, 2014, 8750–8764.
22. Paula A., Amorim D. O., Ribeiro A., Jr, D. C., Peporine N., Nora R., Cristina M., Oliveira C. De, Geraldo M. and Carvalho D. Chemical compounds isolated from Talinum triangulare (Portulacaceae). *FOOD Chem.* 160, 204–208.
23. Vinha A. F., Barreira S. V. P., Castro A., and Machado M. Comparison Between the Phytochemical and Antioxidant Properties of Plants Used in Plant Infusions for Medicinal Purposes. *J. Agric. Sci.* 5, 2013, 11–19.
24. Maharjan B. L., and Baral B. Antioxidant Capacity and Phenolic Content of Some Nepalese Medicinal Plants, 2013, 2013, 1660–1665.
25. Hasan S. M. R., Hossain M., Akter R., Jamila M., Mazumder E. H., and Rahman S. DPPH free radical scavenging activity of some Bangladeshi medicinal plants. *J. Med. Plants Res.*3, 2009, 875–879.
26. Khansari N., Shakiba Y., and Mahmoudi M. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Pat. Inflamm. Allergy Drug Discov.* 3, 2009, 73–80.
27. Gutteridge J. M., Rowley D. A, and Halliwell B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of "free" iron in biological systems by using bleomycin-dependent degradation of DNA. *Biochem. J.* 199, 1981, 263–265.
28. Khan R. A., Khan M. R., Sahreen S., and Ahmed M. Evaluation of phenolic contents and antioxidant activity of various solvent extracts of Sonchus asper (L.) Hill. *Chem. Cent. J.* 6, 2012, 12.
29. Prior R. L., Wu X., and Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53, 2005, 4290–4302.
30. Huang D., Boxin O. U., and Prior R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* 53, 2005, 1841–1856.
31. Aurand L. W., Boone N. H., and Giddings G. G. Superoxide and Singlet Oxygen in Milk Lipid Peroxidation. *J. Dairy Sci.* 60, 1977, 363–369.
32. Korycka-Dahl M., and Richardson T. 8) Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acids. *J. Dairy Sci.* 61, 1978, 400–407.

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