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S Sujatha
Department of Botany,
Bharathiar University,
Coimbatore, Tamil Nadu, India

Dr. T Sekar
Department of Botany,
Bharathiar University,
Coimbatore, Tamil Nadu, India

M Tamilarasan
Department of Biochemisty, Dr. N.G.P. Arts and Science college (Autonomous), Kalapatti Road,
Coimbatore, Tamil Nadu, India

Correspondence
S Sujatha
Department of Botany,
Bharathiar University,
Coimbatore, Tamil Nadu, India

Phytochemicals, antioxidant activities and GC-MS analysis of stem extracts *Toddalia asiatica* (Linn.) lam

S Sujatha, Dr. T Sekar and M Tamilarasan

Abstract
Toddalia asiatica belongs to the family of Rutaceae, stem extract of the plant, collected from Maruthamalai in Hill Western Ghats. Stem was investigated by established *in vitro* studies. Based the material and method quantitative determination of total phenolics, total flavonoids, and various *in vitro* antioxidant activities (DPPH, superoxide radical, reducing power, phosphomolybdenum and metal chelating ability) of methanolic extract was carried out using colorimetric methods. Results are evaluated as higher in stem of *T. asiatica* and recorded antioxidant as well as phytochemical quantitative of total phenol and flavonoid. The plant *T. asiatica* methanolic extract of stem showed greater antioxidant activity of IC_{50} for DPPH assay (49.29 µg/ml) and compare to other extract, reducing power activity stem in methanol (0.647 EDTAE /100g extract), better in phosphomolybdenum reduction (57.4 mg/g acetone extract) and higher superoxide radical scavenging activity in methanolic extract (90.34%). However, the better metal chelating ability was shown by the methanol extracts of stem (4.30 EDTAE/100g) compared to other solvent extracts. The result indicates antioxidant and total phenol activity potential of *T. asiatica*, and concluded based of the GC-MS analysis of bioactive compounds.

Keywords: *Toddalia asiatica*, phytochemical screening, antioxidant activity and GC-MS analysis

1. Introduction
Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants [1]. Free radicals are nature of reactive oxygen species (ROS), which contain all highly reactive, oxygen-containing molecules. Such types of ROS contain the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and different lipid epoxides. These free radicals may either be performed by physiological or biochemical method or by pollution and other endogenous cause. All these free radicals are productive and responding with layer lipids, nucleic acids, proteins and chemicals and other little particles, ensuing in cell damage [2]. These antioxidants may help to relieve oxidative stress, i.e. preventing free radicals from damaging biomolecules such as proteins, DNA, and lipids [3]. The antioxidant activity of extracts of several plants, including their leaves, bark, roots, fruits, and seeds has been extensively studied [4].
Living organisms have antioxidant defence systems that assure against oxidative damage by replacement or removal of spoiled the molecules [6]. The term 'antioxidant' assign to the activeness of numerous vitamins, minerals and phytochemicals which supply strength opposing the damage caused by ROS [7]. A great number of aromatic, medicinal, spice and other plants include chemical compounds express antioxidant character. Oxidative process is one of the most important shows for producing free radicals in foods, drugs and balanced in living systems [8].

2. Material and Methods
2.1 Plant material
The stem parts of *Toddalia asiatica* was collected from Maruthamalai hills, a tail of Western Ghats, during December 2015. The collected plant material was identified and authenticated by Botanical survey of India, Southern Circle, Coimbatore (NO.BSI/SRC/5/23/2014-15/ Tech. /2221) and the voucher specimen has been deposited in Bharathiar University Herbarium, Department of Botany, Coimbatore. The collected fresh plant stem were cleaned thoroughly with running tape water to remove dust and dried in shade for a week at room temperature (30 ^{o} C). The powder was kept in airtight container.

2.2 Plant extracts preparation

The powder of the plant material was extracted by Soxhlet extractor by using successive solvent with petroleum ether, chloroform, acetone and methanol. Each time before extracting the thimble was dried in hot air oven under 40 ºC. The crude extract dissolvable concentrates were thought by rotating vacuum evaporator and after that air dried. The air dried concentrate was weighed. The crude extract yield was noted.

2.3 Quantification of total phenolics, tannins and flavonoids

2.3.1 Quantification of total phenolics and tannin

The total phenol content was determined according to the method described by (Makkar, 2003) [8]. 100 µL aliquots for plants extracts (5mg/ mL) were taken in the test tubes and made up to the volume of 1mL with distilled water. Then 500µl of Folin - Ciocalteu reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added basically in each tube. forthwith vortex the reaction mixture, then the test tubes were located in dark room for 40 min and after the absorbance were recorded at 725 nm against blank. Response blend without plant remove was taken as clear. The examination was performed in triplicate and the outcomes were communicated as Gallic acid equivalents.

Investigated the extract use the tannins were estimated after analysis with polyvinyl polypyrrolidone (PVPP) (Makkar, 2003) [8]. 0.75mg of PVPP was weighed into a 2 mL eppendorf tube and to this 900 µL distilled water and then 150 µL of the sample extracts were added. The content was vortexed and kept in the test tube at 4 ºC for 4hrs. Then the sample was centrifuged at 4000 for 10 minutes at room temperature and the supernatant was collected. This supernatant has just basic phenolics other than the tannins (the tannins would have been encouraged alongside the PVPP). The phenolic substances of the supernatant were measured and show up as the substance of non-tannin phenolics. From the above results, the tannin substance of the example was figured as takes after:

Tannin (in percentage) = Sum of phenolics (in percentage) - Non tannin phenolics (in percentage)

2.3.2 Quantification of total flavonoids

The flavonoid content of the considerable number of concentrates were evaluated as it goes about as a noteworthy antioxidant in plants diminish oxidative anxiety. Assessed according to portrayed by (Zhishen *et al.,* 1999) [9]. Initially 300 µL of all the plant extracts were taken in different test tubes. To each extracts 2 mL of distilled water was added. Then 150 µL of $NaNO_2$ was added to all the test tubes followed by incubation at room temperature for 6 minutes. After incubation 150 µL of $AlCl_3$ (10%) was added to all the test tubes. The test tubes were incubated for 6 minutes at room temperature. Then 2 mL of NaOH was added to all the test tubes which were made up to 5 mL using distilled water. The contents in all the test tubes were vortexes well and they were allowed to stand for 15 minutes at room temperature. The pink colour developed due to the appearance of flavonoids was read spectrophotometrically at 510 nm. The amount of flavonoids was calculated as rutin equivalents.

2.4 *In vitro* antioxidant studies

2.4.1 DPPH radical scavenging activity

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$),according to the method of Blois, (1958) [10]. Plants extracts at various concentrations (20 - 100 µl) was added to 3 mL of 0.1 mM methonolic solution of $DPPH^{\cdot}$ and allowed to stand for 20 min at 27 ºC. The absorbance of the sample was measured at 517 nm. Radical scavenging activity of the sample was expressed as IC_{50}. Concentration of DPPH by 50% (IC_{50}) under the experimental condition was determined. Methanol was served as blank and solution without extract served as control. The mixture of methanol, DPPH and standard (ascorbic acid) served as positive control. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula. More significantly the IC_{50} of the extracts were also calculated.

2.4.2 Phosphomolybdenum assay

The antioxidant power of the extracts has been assessed with the phosphomolybdenum reduction assay according to (Prieto *et al*., 1999) [11]. The assay was based on the reduction of the extract and subsequent formation of a complex (Green colour). 0.5 ml of extract combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was incubated at 95 ºC for 90 minutes. The absorbance of the mixture was measured at 695 nm using spectrophotometer, it was against the blank. The results were calculated in ascorbic acid equivalents/100g extract.

Percentage of Phosphomolybdenum = (Control OD – Sample OD / Control OD) ×100

2.4.3 Reducing power assay

The reducing power of plant in different solvent extracts of was determined by the method reported by (Oyaizu, 1986) [12]. 100 µl of extract was taken in 2.5 ml of 0.2M phosphate buffer (pH 6.6) was added. To this, 2.5 ml of 1% Potassium ferricyanide solution was added and the mixture was incubated at 50 ºC for 20 minutes. After the incubation, 2.5 ml of 10% TCA was added. The content was centrifuged at 3000 rpm for 10 minutes. The upper layer of the supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance of the reaction mixture was measured spectrophotometer at 700 nm.

% reducing power = (Control OD – Sample OD / Control OD) × 100

2.4.4 Assay of superoxide radical (O_2^{*-}) scavenging activity

The assay was based on the capacity of the sample extract to inhibit formazan formation by scavenging superoxide radicals generated in riboflavin- light-NBT system (Beauchamp and Fridovich, 1971) [13]. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 2.33µg riboflavin and 12 mM EDTA, and 11.55 g NBT. Reaction was started by illuminating the reaction mixture with of sample extracts (100 µl) for 90 seconds. Reaction mixture with extract kept in dark served as negative control while the mixture without extract was taken as blank. Immediately after illumination, the absorbance was measure at 590 nm. The activity was compared to ascorbic acid. The percentage inhibition of superoxide anion generation was calculated using the following formula:

Percentage of inhibition = (Control OD – Sample OD / Control OD) × 100

2.4.5 Metal chelating activity

Iron II chelating activity was measured by the inhibition of the formation of Iron-(II)-ferrozine complex after pre-incubation of the sample. The Fe^{+} was monitored by measuring the formation of ferrous iron–ferrozine complex against methanol blank at 562nm.The chelating of ferrous ions by various extracts in plant was estimated by the method of Dinis *et al*.,(1994) [14]. The chelating of ferrous ions by various extracts of *T.asiatica* stem extract was estimated. Initially, about 100µl the extract samples were added to 50µl of 2 mM $Fecl_2$ solution. Then the reaction was initiated by the addition of 200µl of 5mM ferrozine and the test tubes were vortexes well and left standing at room temperature for 10 minutes. The reaction mixture containing deionized water in place of sample was considered as the negative control absorbance of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). EDTA was against the standard metal chelating agent and the results were expressed as mg EDTA equivalents/g extract chelate the ferrous ion was calculated by,

Percentage chelation = [1-(ABS sample/ABS control)] ×100

Ec_{50} value (mg extract / mL) is the effective concentration at which ferrous ions were chelated 50% by the extract.

2.5 Gas chromatography Mass Spectroscopy

Preparation of extract:-1 ml of methanol extract of *T. asiatica* stem of methanol extract was employed for GC/MS.

2.5.1 Instruments and chromatographic conditions

GC-MS analysis was carried out on THERMO GC - TRACE ULTRA VER: 5.0, equipment with run time of 37.53mins and the mass spectrometry (MS) was carried out by using THERMO MS DSQ II equipment. Auto samples and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column DB5-MS capillary standard non polar column, Dimension (30Mts, ID: 0.25 mm, FILM:0.25µm) composed of 5%phenyl polysilphenylene-siloxane and its operating in electron impact mode at 80ev, helium (99.999%) was used as carrier gas at a constant flow of 1.0 ML/min and an injection volume of 1 micro liter was employed (split ratio of 10.1) injector temperature 250 ℃, ion-source temperature 280℃. The oven temperature was programmed from oven temperature 70 ℃ raised to 260 ℃ at 6 ℃/min up to 450 ℃. Mass spectra were taken at 80ev, a scan interval of 0.5 second and fragment from 40 to 550Da.

2.5.2 Identification of components

The mass spectrum of the unknown compound was compared with spectrum of the known components stored in the NIST (National institute of standard technology) library. The name, molecular weight and structure of the components of the test material were ascertained.

2.5.3 Statistical analysis

All analyses were carried out in triplicates. The results of scavenger activity and total phenolic and total flavonoids contents were performed from the averages of all samples reading Mean ± SD used Excel 2003. Analysis of antioxidant variance using one-way ANOVA was performed to test the significance of differences between means at the 0.05 level of significance using the statistical analysis software, SPSS (IBM-SPSS statistic version 20).

3. Results and Discussion

3.1 Quantification of total phenolic, tannin and flavonoid

The results of total phenolics and tannin contents are showed in Table 1. Methanol extracts of *T. asiatica* stem revealed highest phenolic (55.9 ± 1.02 GAE/100g) when estimation of tannin methanol extracts of *T. asiatica* stem revealed highest amount shows (2.5 ± 1.05 GAE/100g).The results of flavonoid content are presented in Table 1. In this estimation of the methanol extract of *T. asiatica* stem revealed maximum amount of flavonoid content (46.2 ± 2.03/100g).

Table 1: Determination of phenolic, tannin and flavonoid content in stem extract of *T. asiatica:*

S. No	Stem Extract	Total phenol (GAE mg/100g)	Tannin (GAE mg/100g)	Total Flavonoid (RU mg/100g)
1.	Pet ether	38.5 ± 1.80^{d}	2.8 ± 2.18^{c}	17.3 ± 8.74^{c}
2.	Chloroform	44.6 ± 0.55^{c}	1.1 ± 3.12^{d}	12.3 ± 3.51^{d}
3.	Acetone	52.9 ± 0.58^{b}	2.5 ± 1.05^{b}	43.2 ± 1.20^{b}
4.	Methanol	55.9 ± 1.02^{a}	3.1 ± 0.26^{a}	46.2 ± 2.03^{a}

*Values are man of three replicate determinations (n = 3) ± standard deviation. Mean values followed by different super scripts in a column are significantly different (P < 0.05). GAE – Gallic acid equivalence, RU – Rutin equivalence.

3.2 Antioxidant activity

3.2.1 Radical scavenging activity using DPPH· method

In *T. asiatica* stem extract in acetone extract shows (41.02µg/ml) the higher inhibitory activity, the inhibitory percentage of standard natural antioxidant – Vitamin-C was found to be much better than that of plant extracts.

3.2.2 Phosphomolybdenum assay

Phosphomolybdenum assay is successfully used to determine the ability of extracts to reduce Mo (VI) to Mo (V) and subsequent formation of green phosphate /Mo(V) complex at an acid pH. The total antioxidant capacity of different solvent extracts of stem of *T. asiatica* was analyzed and shown in Figure 2. In *T. asiatica* stem showed higher activity in most of its solvents compared to the extracts, Methanol extract of stem (57.4 mg/g; extract).

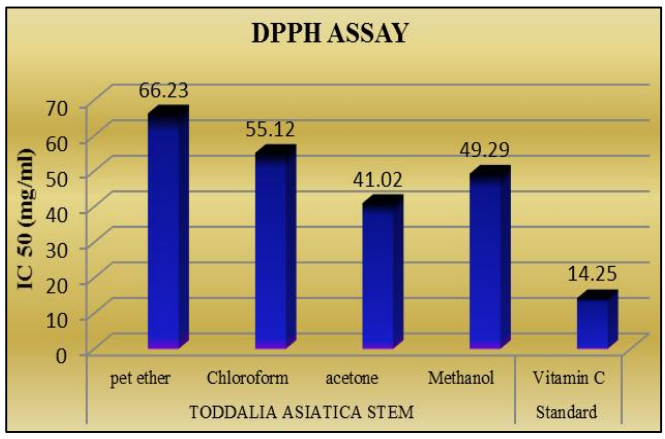


Fig 1: DPPH· scavenging activity of *T. asiatica* stem extract

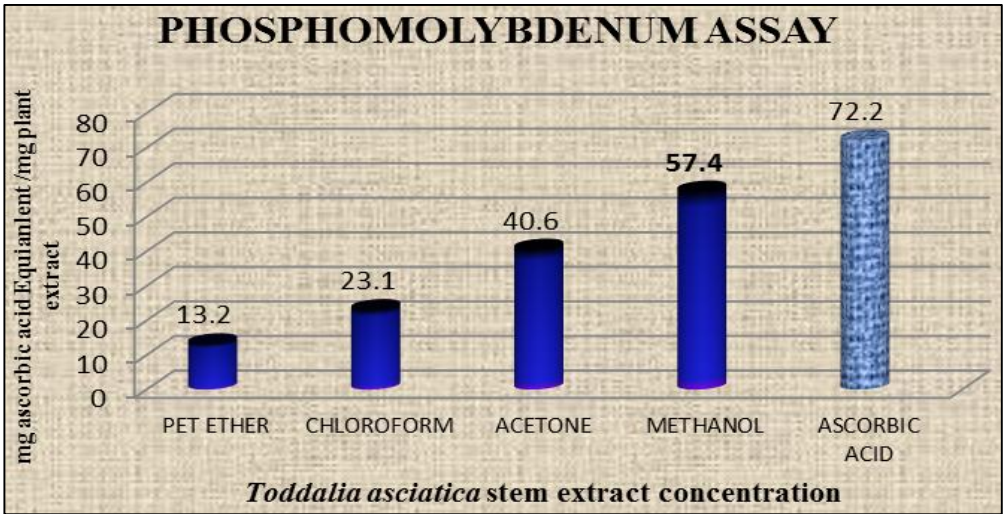


Fig 2: Phosphomolybdenum activity of *T. asiatica* stem extract

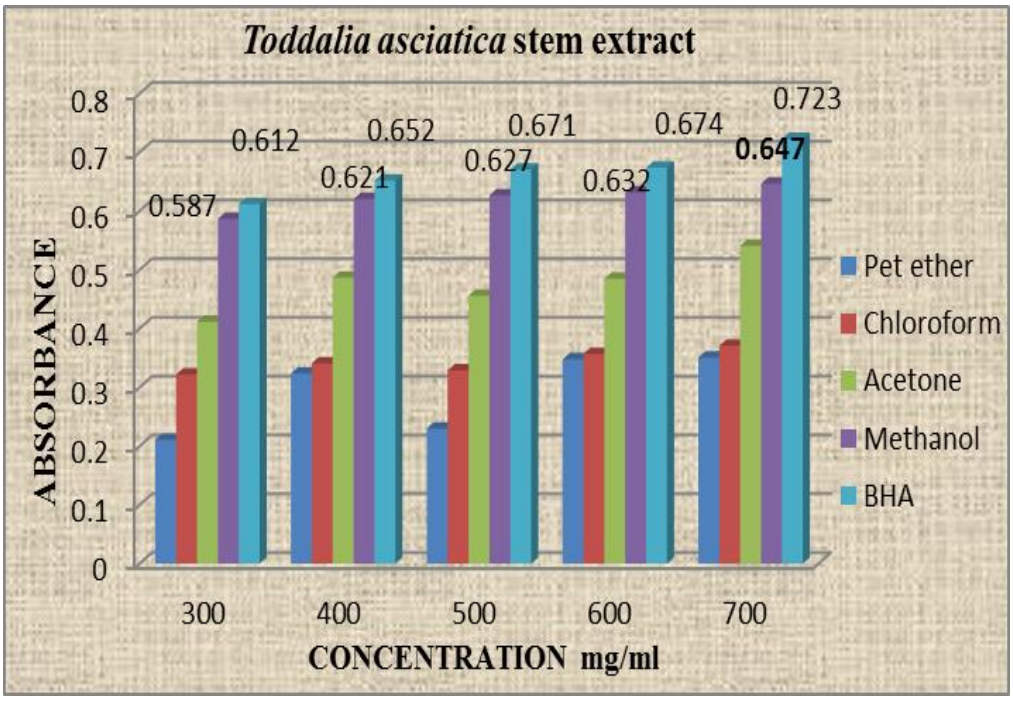


Fig 3: Reducing power of *T. asiatica* stem extract

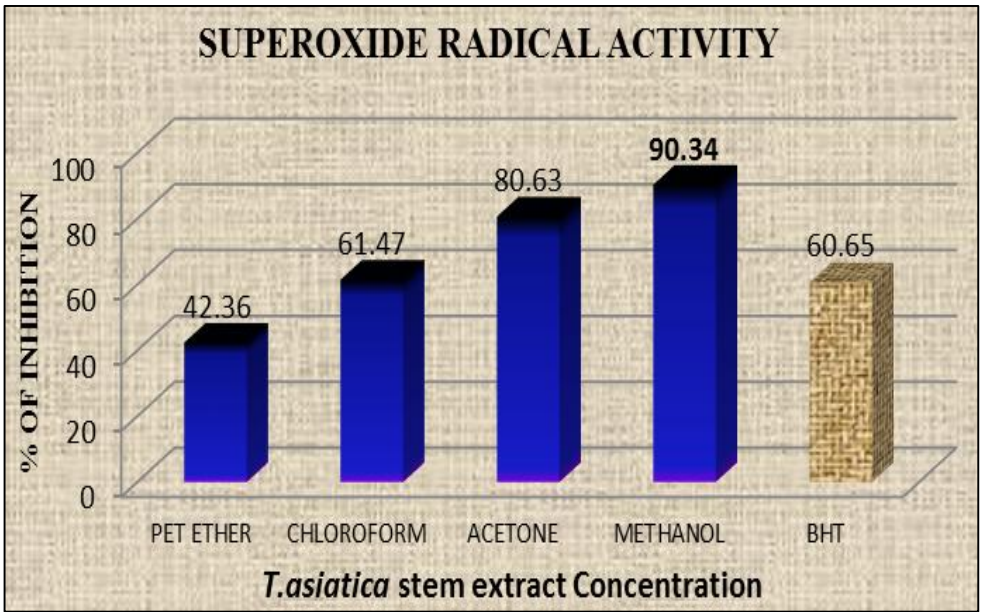


Fig 4: Superoxide radical scavenging activity of *T. asiatica* stem extracts

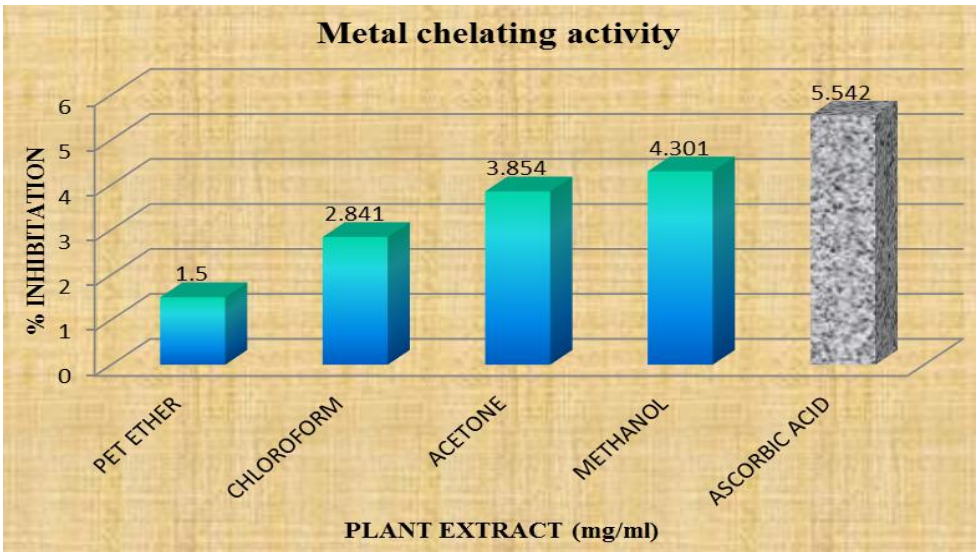


Fig 5: Metal chelating activity of *T. asiatica* stem extracts

3.2.3 Reducing power assay

The presence antioxidant purpose the reduction of $Fe3^{+}$ / ferricyanide complex to the ferrous form. The reducing power of stem and leaf extracts of *T. asiatica* was calculated and the results were presented in the Figure 5. The *T. asiatica* methanol extract in stem (0.647%) shows higher reducing power compared to other extracts.

3.2.4 Superoxide radical (O_2^{*-}) scavenging activity

The superoxide anion radical scavenging activities of *T. asiatica*, stem is shown in Figure 6. The extracts were found to be an efficient scavenger of superoxide radical generated in riboflavin- NBT- light system *in vitro*. The methanol extract of *T. asiatica* stem showed higher superoxide radical scavenging activity (90.34%) compared to other solvent extracts at a concentration of 100µg/ml.

3.2.5 Metal chelating activity

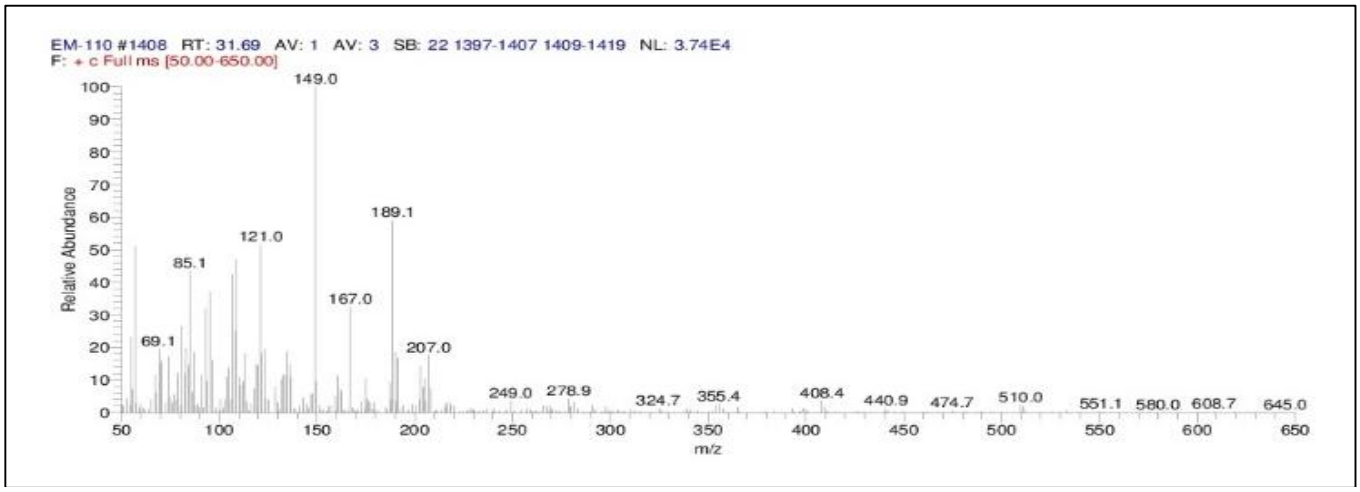
The antioxidants present in plant extract forms a coordinate complex with the metal ions (chelating activity) and inhibit the transfer of electrons. Thus oxidation reaction is arrested and no free radicals are produced. The Fe^{+} chelating capacity of different solvent extracts of *T. asiatica* stem is shown in Figure 7. In *T. asiatica* maximum chelations were observed for the methanol extract of stem (4.30 mg EDTAE/100g) extract.

3.3 GC-MS chromatogram of *Toddalia asiatica* methanol stems extract

In the *T. asiatica* stem extract result shows higher percentage of phytochemicals were namely listed in the table, Lupeol, Betulin (29.38%) are triterpenoid compund, and mainly used in pharmaclogical value of antimicrobial, antiinflammatory, antitumor activity; SOLANESOL medicinal value of antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, and anti-ulcer activities. á-Amyrin trimethylsilyl ether (10.12%); Podocephalol (10.40%). 9-Octadecenoic acid (Z)-, methyl ester (CAS) have antibacterial and Antioxidant activity, Anticarcinogenic,- exist in human blood and urine and serve as endogenous peroxisome proliferator- activated receptor ligand, dermatitigenic flavour activities.

The GC-MS study of the methanolic extract of the stem *T. asiatica* had shown the presence of many phytochemicals which might contribute to the medicinal activity of that plants. The comparison of the mass spectrums with the data base gave more than 90% match as well as confirmatory compound structure match. The GC-MS analysis of the methanol extract resulted many compounds which have diverse use. These Compounds are having so many medicinal purposes as like anti-inflammatory, antibacterial, antifungal, acute toxicity, anti-cancer, skin conditioning properties have been identified.

3.3.1 GC-MS chromatogram of *Toddalia asiatica* methanol stems extract



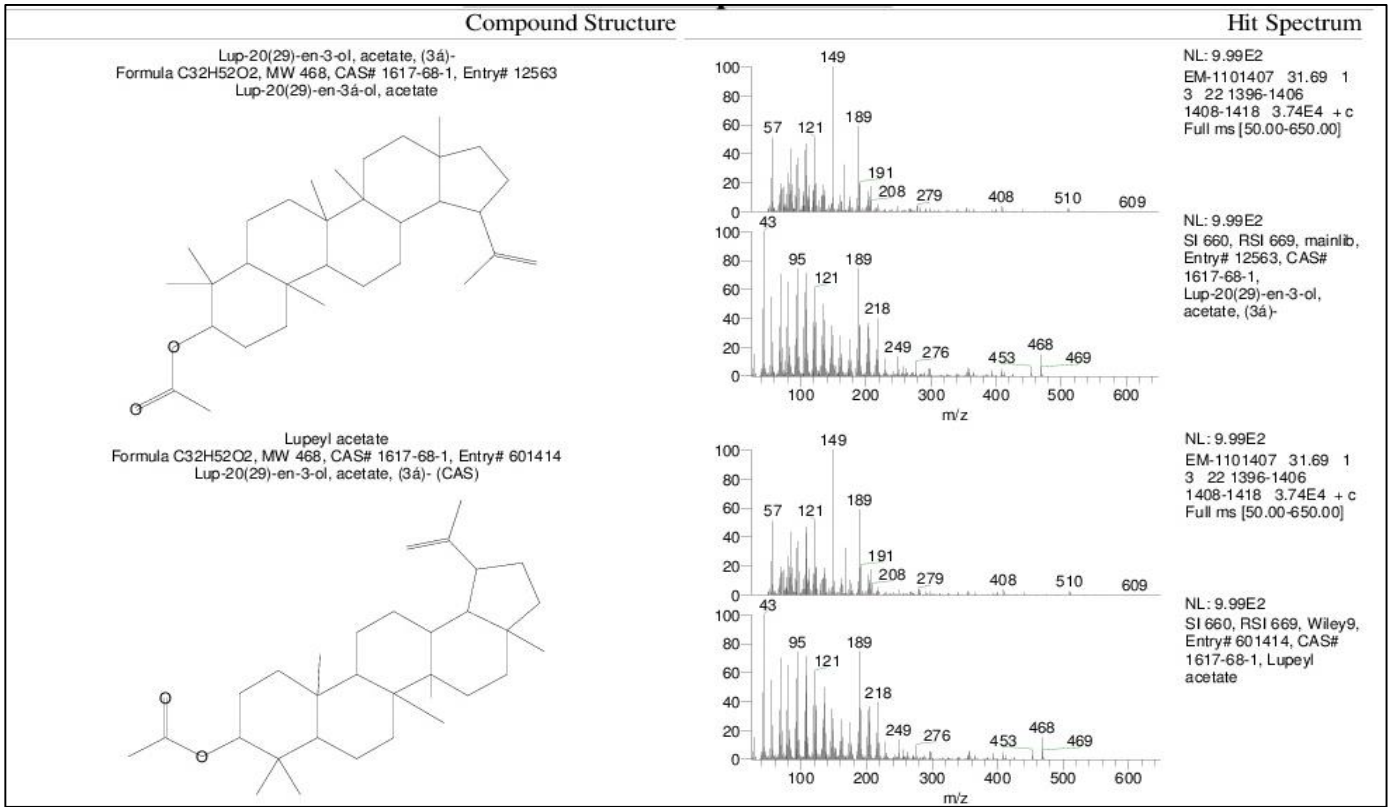


Table 2: GC-MS Identified Components of The Plant *T. asiatica* Stem of Methanolic Extract

S. No	RT (min)	Name of Compound	Molecular Formula	Molecular Weight	AREA%
1.	2.36	Podocephalol	C15H22O	218	10.40
2.	2.27	SOLANESOL	$C_{45}H_{74}O$	630	26.38
3.	2.43	á-Amyrin trimethylsilyl ether	C33H58OSi	498	10.40
4.	2.57	Lactaropallidin	$C_{15}H_{24}O_3$	252	26.38
5.	4.14	ISOCHIAPIN B	$C_{19}H_{22}O_6$	346	2.44
6.	4.41	1-(Benzyloxy)-2-fluoro-2-phenyl-3-(p-toluenesulsulfonyloxy)propane	$C_{23}H_{23}FO_4S$	414	2.90
7.	4.73	1-Tetradecanol (CAS)	$C_{14}H_{30}O$	214	1.06
8.	5.78	Lupeol	$C_{30}H_{50}O$	426	26.38
9.	5.79	6-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	7.36
10.	6.94	à-CYPERONE	$C_{15}H_{22}O$	218	10.40
11.	7.17	Betulin	$C_{30}H_{50}O_2$	442	26.38
12.	7.62	4-Hydroxy-2-methylpyrrolidine-2-carboxylic acid	$C_6H_{11}NO_3$	145	2.23
13.	7.94	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- (CAS)	$C_{27}H_{52}O_4Si_2$	496	2.44
14.	9.95	9-Octadecenoic acid (Z)-, methyl ester (CAS)	C19H36O2	296	7.36
15.	18.15	Lupeyl acetate	$C_{32}H_{52}O_2$	468	26.38

Table 3: Major phyto compounds and biological activities of *T. asiatica*

No	Name of compound	Nature of compound	*Activity
1	Solanesol	Terpenes	Used in synthesis of high-value biochemicals such as vitamin-K
2	Lupeol	triterpenoid	Anti-inflammatory and anticancer activity
3	Betulin	triterpenoid	Anticarcinomic, Antifeedant, Antiflu, Antiinflammatory, Antitumor, Antiviral, Hypolipemic.
4	Lupeyl acetate	triterpenoid	Anti-cancer, antioxident.

4. Conclusion

The presence studies of the plant *T. asiatica* (L.) can be valuable natural antioxidants, total phenol activity and GC-MS analysis. The first steps towards understanding the nature of the active principles in this medicinal plant and phytochemicals will be helpful for further detailed study. The importance of the study was to identify some of the biological activities of these compounds in *T. asiatica* stem, suggests a contribution from these compounds to pharmacological activity in the future.

5. Acknowledgements

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