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Research Article

EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *TARENNA ASIATICA* (L.) O. KTZE. *EX* K. SCHUM.

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ABSTRACT

Objective: The study was aimed to analyze the phytochemical, antioxidant and antibacterial potentials of *T. asiatica*.
Methods: The leaves, barks and flowers extracts were analyzed for total phenolic and flavonoid contents. Antioxidant activities were evaluated using DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging, FRAP, phosphomolybdenum reduction, metal chelating, nitric oxide radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging and lipid peroxidation assays. Antibacterial activity was examined using agar well diffusion method against pathogenic microorganisms.
Results: The total phenolic and flavonoid contents were found to be higher in acetone extract of leaves (57.21 g GAE/100 g and 619.67 mg RE/g respectively). Antioxidant assays revealed that leaves acetone extract possesses significant (p<0.05) DPPH$^{\bullet}$ scavenging capacity (IC_{50}: 20.38 µg/mL), ABTS$^{\bullet+}$ scavenging activity (10435.44 µM TE/g), ferric reducing activity (152.13 mM Fe(II)E/mg), phosphomolybdenum reduction (417.93 mg AAE/g) and metal chelating activity (20.85 g EDTAE/100 g). At a concentration of 200 ug/mL, the leaves acetone extract also showed higher nitric oxide radical (49.22%), superoxide radical (73.63%) and hydroxyl radical (69.04%) scavenging activities and inhibition for lipid peroxidation (57.38%). Leaves and flowers acetone extracts inhibited the growth of *S. dysenteriae*, *B. subtilis* and *S. boydii* with an inhibition zone ≥12 mm. MIC of flowers acetone extract was found to be 20 µg/mL against both *B. subtilis* and *S. boydii*.
Conclusion: *T. asiatica* contains considerable phenolic and flavonoid contents which is responsible for the evident antioxidant and antimicrobial activities. These findings validate that *T. asiatica* can be a natural antioxidant and antibacterial source which will address medical security.

Keywords: Antimicrobial, Antioxidant, Flavonoid, Free radical, Phenolic, Reactive oxygen species, *Tarenna asiatica*

INTRODUCTION

Medicinal plants have enormous therapeutic potential to heal many infectious diseases by avoiding many side effects [1]. The secondary metabolites such as phenolics, flavonoids, alkaloids, iridoids etc. present in the plant extracts, generally produced by plants for their defence mechanisms have been implicated in the therapeutic properties of most medicinal plants [2] Over-production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in humans can result in disease development and oxidative damage to nucleic acids, proteins, and lipids. The lack of antioxidants to quench excess reactive free radicals leads to oxidative stress and thereby cancer, cardiovascular, neurodegenerative, inflammatory and Alzheimer's diseases [3]. Recent studies revealed that synthetic antioxidants can be toxic and expensive and this generated a need to identify natural and probably safer sources of antioxidants [4].

Antibacterial activity is the ability of a substance to inhibit or kill bacterial cells. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbes has led to the screening of several medicinal plants for their potential antimicrobial activity [5]. Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. Therefore, there is need to search new infection-fighting strategies to control microbial infections and most concern about the alarming increase in the rate of infection by antibiotic-resistance microorganisms [6]. Thus current research focuses the development of antimicrobials of plant origin which can be used as an alternative to synthetic drugs.

Tarenna asiatica which belongs to the family Rubiaceae has a significant position among medicinal plants by virtue of its several phytotherapeutic values. The ethnobotanical reports reveal that *T. asiatica* has been used for treatment of several disorders such as boils, external ulcers and sores [7]. The leaves possess wound healing property so that the leaves infusion is given orally in plough injuries [8, 9] and it is used as antidote [10]. *T. asiatica* is used as one of the ingredients of preparation for paralysis by the Malayali tribe in Nalamankadai, Eastern ghats, India; in which the young leaves of the plants were ground, made into paste and applied externally to affected portion for two to three months [11]. Sahu *et al*. reported the wound healing property of leaves paste of *T. asiatica* mixed with turmeric powder which is applied externally on the head of the children for curing wounds [12]. Moreover, the anti-inflammatory effect of aqueous suspension of leaves powder rather than their extracts was reported by Amutha and coworkers through carrageenan induced lung inflammation in rats [13].

T. asiatica leaves are also used in skin diseases whereas their fruits are smashed and applied to boils to promote suppuration [14, 15]. Besides, the classical notes on the medicinal property of its fruits points out the application of fruit juice on the eyelids to arrest infection [16, 17]. The tribal population in the hilly tracts of Eastern Ghats in Andhra Pradesh uses the stem barks of *T. asiatica* for vomiting [18]. Even though *T. asiatica* possesses enormous medicinal values, less effort was made to scientifically evaluate their potentials. Therefore, the present research work has been carried out to scientifically validate the antioxidant and antimicrobial potentials of *T. asiatica*.

MATERIALS AND METHODS

Collection and identification of plant material

Fresh leaves, barks and flowers of *T. asiatica* were collected during the month of May 2012 from Maruthamalai hills, Coimbatore district of Tamil Nadu, India. The taxonomic identity of the plant was confirmed from the Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu (No: 293). The fresh plant materials

were washed under running tap water to remove the surface pollutants and were air dried under shade. Then they were separately homogenized into fine powder using mixer and used for further studies.

Chemicals and standard drugs

The chemicals such as 2,2-diphenyl-1-picryl-hydrazyl (DPPH•), potassium persulfate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Sodium nitroprusside, Butylated hydroxy anisole (BHA), Butylated hydroxy toluene (BHT), rutin, gallic acid, ferrous chloride, ferric chloride, ferric cyanide, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), hydrogen peroxide, ethylene diamine tetraacetic acid (EDTA) disodium salt, N-(1-naphthyl) ethylene diamine dihydrochloride, riboflavin and Muller Hinton agar medium (MHA) were obtained from HiMedia (Mumbai, India) and Sigma Aldrich (Bengaluru branch, India). All the chemicals and solvents used were of the highest purity and analytical grade.

Preparation of extracts

The freshly collected plant materials were washed thoroughly in tap water, shade dried at room temperature (25°C), powdered and used for solvent extraction. The plant samples were successively extracted with petroleum ether (for disposing lipid and pigments), chloroform, acetone and methanol using soxhlet apparatus and the air dried residue were further extracted with hot water by the method of maceration for 24 h. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. The solvents were evaporated using a rotary vacuum-evaporator (Yamato RE300, Japan) at 50°C and the remaining solvent was removed by lyophilisation (VirTis Benchtop K, USA). The extract recovery in different solvents was expressed as percent of the plant sample dry matter. The freeze-dried extracts thus obtained were dissolved in the respective solvents at the concentration of 1 mg/mL and used for antioxidant and antimicrobial studies.

Quantification assays

Quantification of total phenolics

The total phenolics of *T. asiatica* were determined according to the method described by Makkar [19]. About 50 µL of plant extracts were taken into a series of test tubes and made up to 1 mL with distilled water. A test tube with 1 mL of distilled water served as the blank. Then, 500 µL of Folin – Ciocalteau Phenol reagent (1 N) was added to all the test tubes including the blank. After 5 minutes, 2.5 mL of sodium carbonate solution (20%) was added, vortexed well to mix the contents, and incubated in dark for 40 minutes. The formation of blue colour in the incubated test tubes indicated the presence of phenolics which was read at 725 nm against the reagent blank. Gallic acid was used as standard to plot the graph and the results were expressed as gallic acid equivalents (GAE). The analyses were performed in triplicates.

Quantification of flavonoids

The method of Zhishen *et al*. was followed for the quantification of flavonoids in the extracts [20]. About 2 mL of distilled water was added to 500 µL of different plant extracts taken in test tubes. The blank test tube contained only 2.5 mL of distilled water. Then, 150 µL of 5% $NaNO_2$ was added to all the test tubes followed by incubation at room temperature for 6 minutes. After incubation, 150 µL of 10% $AlCl_3$ was added to all the test tubes including the blank. Again, the test tubes were incubated for 6 minutes at room temperature. Then, 2 mL of 4% 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 vortexed well and allowed to stand for 15 minutes at room temperature. The pink colour developed due to the presence of flavonoids was read spectrophotometrically at 510 nm. The flavonoid - rutin was used as the standard for plotting the calibration curve. All the experiments were done in triplicates and the results were expressed in rutin equivalents (RE).

***In vitro* antioxidant assays**

DPPH• scavenging activity

The antioxidant activity of *T. asiatica* extracts were determined according to the method of Braca *et al*. [21], in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The extract aliquots were taken and made upto 100 µL with methanol. About 3 mL of a 0.004% methanolic solution of DPPH was added to all the test tubes containing samples and standards. Negative control was prepared by adding 100 µL of methanol to 3 mL of DPPH solution. The tubes were allowed to stand for 30 minutes at 27°C. The absorbance of the sample was measured at 517 nm against the blank (methanol). Radical scavenging activity of the samples was expressed as IC_{50} which is the concentration of the sample required to inhibit 50% of DPPH• concentration.

ABTS•+ scavenging activity

ABTS radical cation decolourization assay was performed for the samples according to the method of Re *et al*. [22]. ABTS•+ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 hours at room temperature. Prior to assay, this solution was diluted with ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance of 0.700 ± 0.02 at 734 nm. After the addition of 1 mL of diluted ABTS solution to aliquots of sample or Trolox (analog of vitamin E), absorbance was measured exactly 30 minutes after the initial mixing. Triplicate determinations were made and the decolourization was read at 734 nm against the blank (ethanol). The unit of antioxidant activity was calculated as the concentration of Trolox having equivalent antioxidant activity expressed as µM/g sample extracts.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacities of different extracts of samples were estimated according to the procedure described by Pulido *et al*. [23]. FRAP reagent (900 µL), prepared freshly and incubated at 37°C, was mixed with 90 µL of distilled water and 30 µL of test sample or methanol (blank). The test tubes were incubated at 37°C for 30 minutes in a water bath. The FRAP reagent was prepared by mixing 2.5 mL of 20 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$, and 25 mL of 0.3 M acetate buffer (pH-3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm against the reagent blank. Methanolic solutions of known Fe(II) concentration, ranging from 100 to 2000 µM, ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. Equivalent concentration was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of Fe(II) solution.

Phosphomolybdenum reduction assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al.* [24]. Triplicates of 150 µL of sample and different concentrations of standard (ascorbic acid in 1 mM dimethyl sulphoxide) were added with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a test tube. The test tubes were covered with aluminium foil and incubated in a water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. A typical blank solution contained 3 mL of reagent solution and water in place of sample, and it was incubated under the same conditions as the rest of the samples. The results reported are mean values expressed as mg of ascorbic acid equivalents per gram extract.

Metal chelating activity

The chelating of ferrous ions by various extracts of *T. asiatica* was estimated by the method of Dinis *et al.* [25]. Briefly, 1000 µL of samples and BHT (standard) were added to 100 µL solution of 2 mM $FeCl_2$. The reaction was initiated by the addition of 400 µL of 5 mM

ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). The metal chelating capacities of the extracts were expressed as mg EDTA Equivalent/100 g extract.

Nitric oxide radical scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide [26] which interacts with oxygen to produce nitrite ions which can be estimated by use of the Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide). Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with plant extracts (200 µL) and incubated at 25°C for 150 min. A control solution without sample and blank (saline buffer) was conducted in an identical manner. At intervals, samples (0.5 mL) of the incubation solution were removed and diluted with 0.5 mL of Griess reagent (1% sulphanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine was read at 546 nm. The scavenging activity on nitric oxide was calculated as: scavenging activity (%) = $[(A_0 - A_1) / A_0] X 100$; where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract/standard.

Superoxide radical scavenging activity

The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin–light–NBT system [27]. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH-7.6), 20 µg riboflavin, 12 mM EDTA, 0.1 mg NBT and 100 µL of trplicate of sample solution or BHA and BHT (standard). Reaction was started by illuminating the reaction mixture with sample extract for 90 s. Immediately after illumination; the absorbance was measured at 590 nm against the reagent blank (reaction mixture without plant sample). Identical tubes with reaction mixture kept in the dark served as black. The scavenging activity on superoxide anion generation was measured as: scavenging activity (%) = $[(A_0 - A_1) / A_0] X 100$; where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract/standard.

Hydroxyl radical scavenging activity

The scavenging activity of different solvent extracts of *T. asiatica* on hydroxyl radical was measured according to the method of Klenin *et al.* [28]. A triplicate of 200 µl of different solvent extracts were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1mL of DMSO (0.85% V/V) in 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% W/V). 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of color formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage of hydroxyl scavenging activity was calculated by the following formula: scavenging activity (%) = $[(A_0 - A_1) / A_0] X 100$; where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract/standard.

Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media [29]. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (500 µL of 10%, v/v in phosphate-buffered saline pH 7.4) and 200 µL of sample were added to a test tube and made up to 1.0 mL with distilled water. Then, 50 µL of $FeSO_4$ (0.075 M) and 20 µL of L-ascorbic acid (0.1 M) were added and incubated for 1 h at 37°C to induce lipid peroxidation. Thereafter, 0.2 mL of EDTA (0.1 M) and 1.5 mL of TBA reagent (3 g TBA, 120 g TCA and 10.4 mL 70% $HClO_4$ in 800 mL of distilled water) were added in each sample and heated for 15 min at 100°C. After cooling, samples were centrifuged for 10 min at 3000 rpm and absorbance of supernatant was measured at 532 nm. Inhibition (%) of lipid peroxidation was calculated using the equation: % Inhibition = $[(A_0 - A_1) / A_0] X 100$, where, A_0 is the absorbance of the control, and A_1 is the absorbance of the tested sample.

Antibacterial assay

Microorganisms used

The bacterial strains namely *Klebsiella pneumoniae*, *Proteus vulgaris*, *Shigella dysenteriae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella boydii*, *Bacillus subtilis*, *Corneybacterium diphtheriae* and *Staphylococcus aureus* were collected from the Department of Biotechnology, Periyar University, Salem – 636 011, Tamil Nadu. The collected strains were stored in refrigerator at 4°C and sub-cultured in 30 days interval on Muller Hinton agar slants. Each inoculum was adjusted to McFarland standard, equivalent to $1×10^8$ CFU which was used for performing antibacterial tests.

Agar well diffusion method

Antibacterial screening of leaves, barks and flowers extracts of *T. asiatica* was done using agar well diffusion method against selected pathogenic microorganisms with required modifications. For this 25 mL of sterile Muller Hinton agar No. 2 containing the suspension of test organisms (50 µL) was swabbed on the molten MHA plates by using sterile cotton swab. Sample wells were made using sterile cork-borer (5 mm diameter) on each seeded plates and labeled properly. Standard antibiotic chloramphenicol (100 µg/mL) was used as positive control. Sterile DMSO (100%) was used as negative control. Then, approximately 50 µL of each extract (1 mg/mL) and chloramphenicol was separately introduced into wells and allowed to diffuse at room temperature. The plates were kept in incubator at 37°C for 24 hours. After incubation, the diameter of inhibition zone was measured by ruler and recorded in millimeters.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations were estimated by broth dilution technique [30]. The culture tubes were seeded with respective cultures and different concentration of plant extracts (leaves, barks and flowers) was added and incubated at 37°C for 24 hours. The lowest concentration of plant extracts inhibiting the growth of the organism and which did not permitting any visible growth of the inoculated test organism in broth culture, was regarded as the minimum inhibitory concentration (MIC) in each case.

Determination of Minimum Bactericidal Concentration (MBC)

After culturing the test organisms separately in nutrient broth containing various concentrations of the plant extracts, the broth was inoculated onto freshly prepared agar plates to assay for the bactericidal effect. The culture was incubated at 37°C for 24 hours. The lowest concentration of plant solvent extract that does not yield any colony growth on the solid medium after the incubation period was regarded as minimum bactericidal concentration (MBC) [31].

Statistical analyses

All the experiments were done in three replicates and the results were expressed as Mean ± Standard Deviation (SD). The statistical analyses were done by using SPSS version 17.0 by means of one way ANOVA followed by Duncan test for antioxidant studies.

RESULTS

Extract Recovery Percent

The percentage yield of leaves barks and flowers extracts of *T. asiatica* in different solvents are presented in table 1. The better yield was obtained for methanol extraction where the yield percentage was 21.6, 15.48 and 11.78 g for flowers, leaves and barks

respectively. However, the extract recovery percent was found to be lower in the chloroform extracts of leaves (3.19 g), flowers (2.67 g) and barks (1.48 g).

Quantification of total phenolics and flavonoids

The results of total phenolics and flavonoid contents are shown in table 1. Acetone extract of *T. asiatica* leaves revealed highest phenolic content (57.21 g GAE/100 g) followed by acetone (48.26 g GAE/100 g) and methanol (43.98 g GAE/100 g) extracts of barks. In the estimation of flavonoids, the acetone extract of leaves exhibited significant amount of flavonoid content (619.67 mg RE/g) followed by acetone extract of barks (561.00 mg RE /g) and flowers (497.00 mg RE/g).

Table 1: Extract yield, total phenolics and flavonoid contents of *T. asiatica*

Parts	Solvents	Extract yield (g/100 g dried powder)	Total Phenolics (g GAE/100g extract)	Flavonoids (mg RE/g extract)
Leaves	Petroleum Ether	6.12	25.40±1.25	141.33±7.23
	Chloroform	3.19	37.31±2.30^{c}	135.00±2.00
	Acetone	5.19	57.21±4.59^{a}	619.67±19.27^{a}
	Methanol	15.48	20.62±3.51^{b}	426.00±5.29
	Hot Water	8.91	16.93±3.93	446.33±2.52^{d}
Barks	Petroleum Ether	2.73	18.86±0.71	106.00±1.73
	Chloroform	1.48	28.62±1.97	88.67±2.52
	Acetone	1.83	48.26±5.32^{b}	561.00±10.24^{b}
	Methanol	11.78	43.98±1.80^{b}	317.0±8.72
	Hot Water	6.63	34.74±0.55^{c,d}	117.67±3.51
Flowers	Petroleum Ether	4.48	3.89±1.70	351.33±13.65
	Chloroform	2.67	6.77±2.64	359.33±5.13
	Acetone	4.1	21.73±2.17	497.00±10.09^{c}
	Methanol	21.65	16.49±5.33	301.33±6.66
	Hot Water	9.52	30.88±3.67^{d}	141.00±2.65

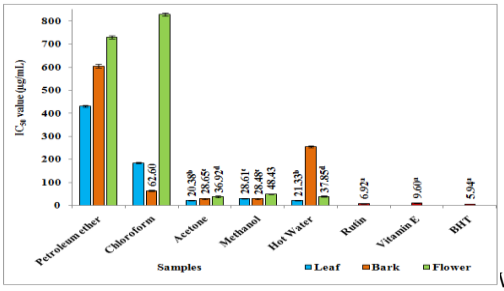
Values are mean of triplicate determination (n=3) ± standard deviation; GAE - Gallic Acid Equivalents; RE - Rutin Equivalents
Statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$ in each column

Antioxidant assays

DPPH radical scavenging activity

The free radical-scavenging activities of different parts of *T. asiatica* samples along with standards such as rutin, vitamin E and BHT were determined by the DPPH radical scavenging assay and the results are represented in figure 1. The lower IC_{50} values indicate higher DPPH radical scavenging activities. Generally the acetone and methanol extracts of all the parts showed significant reduction of DPPH radical. However, the highest free radical scavenging activity was exerted by acetone and hot water extracts of leaves and the IC_{50} values were 20.38 and 21.33 µg/mL respectively.

Figure 1: DPPH radical scavenging activity of *T. asiatica*



Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$

ABTS radical cation scavenging activity

The trolox equivalent antioxidant capacity was measured using ABTS radical cation decolourisation assay; one of the most frequently employed methods for antioxidant activity. The results of ABTS radical cation scavenging activities of different extracts of leaves, barks and flowers of *T. asiatica* are shown in table 2. Among the different solvent extracts, the acetone extracts of leaves and barks revealed appreciable level of trolox equivalent antioxidant activity (10435.44 and 7269.71µM TE/g extract respectively). In connection to that the methanol and hot water extracts of leaves also exhibited higher activity (6540.71 and 5376.34 µM TE/g extract respectively).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing capacities of *T. asiatica* extracts are shown in table 2. The ferric reducing activity observed for the plant extracts ranged between 6.56 to 152.13 mM Fe(II)E/mg extract. The significant reducing capacities were shown by the acetone of extracts of all the parts and the decreasing order of activity for the parts was leaves > barks > flowers. It was also seen that the hot water extract of leaves also showed higher activity which was estimated to be 119.05 µM Fe(II)E/g extract.

Phosphomolybdenum reduction assay

The total antioxidant capacity of different solvent extracts of leaves, barks and flowers of *T. asiatica* were analyzed and are shown in table 2. Among different parts used, leaves showed higher activity in most of its solvents compared to the extracts of barks and flowers. Acetone extract of leaves and flowers (417.93 and 337.11 mg AAE/g extract) exhibited the highest phosphomolybdenum reduction compared to other solvent extracts. The methanol extracts also possessed antioxidant capacities which were 233.02, 224.01 and 221.20 mg AAE/g extract for barks, leaves and flowers respectively.

Metal chelating activity

The iron chelating activity of different extracts of *T. asiatica* are shown in table 2. The significant chelating activity was observed for the acetone extract of leaves (20.85 g EDTAE/100 g extract) followed by the acetone extracts of flowers and barks 15.03 and 10.07 g EDTAE/100 g extract respectively). Moreover, the methanol extract of leaves also revealed its potential in chelating iron and the activity was 14.14 g EDTAE/100 g extract. On the other hand, the hot water as well as non polar extracts were not able to chelate iron and thereby failed to reduce the colour formation by iron-ferrozine complex.

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity of different solvent extracts of all the samples are presented in figure 2. The higher nitric oxide radical scavenging activity was exerted by acetone extract of leaves (49.22%) followed by that of barks (44.62%) at a concentration of 200 µg/mL. However, the methanol and hot water extract of all the parts revealed comparable scavenging activities. Apart from the sample data, the standards viz. rutin and BHT exhibited significantly different activity even at a concentration of 100 µg/mL (65.00 and 58.78% respectively).

Superoxide radical scavenging activity

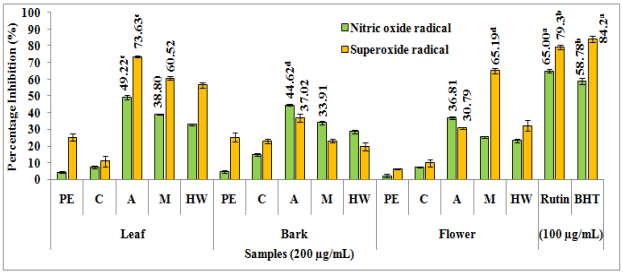
The results of superoxide anion scavenging activities of different extracts of *T. asiatica* are shown in figure 2. The results showed that acetone extract of leaves (73.63%) have highest superoxide radical scavenging activity compared with other solvent extracts of other parts of the plant at a concentration of 200 µg/mL. Among the different extracts of flowers, significant activity was shown by methanol extract and was 65.19%. The scavenging activity was also compared with that of natural (rutin) and synthetic (BHT) antioxidants.

Table 2: ABTS$^{\bullet+}$ scavenging, FRAP, Phosphomolybdenum reduction and Metal chelating activities of *T. asiatica*

Parts	Solvents	ABTS$^{\bullet+}$ scavenging (µM TE/g extract)	FRAP (µM Fe(II)E/g extract)	Phosphomolybdenum reduction (mg AAE/g extract)	Metal chelating (g EDTAE/100 g extract)
Leaves	Petroleum Ether	300.37±77.33	7.80±0.06	31.38±1.01	0.71±0.04
	Chloroform	870.74±53.58	6.56±0.61	115.94±0.41	0.44±0.34
	Acetone	10435.44±23.38^{a}	152.13±0.74^{a}	417.93±6.08^{a}	20.85±0.52^{a}
	Methanol	6540.71±53.58^{c}	93.78±0.32^{d}	224.01±8.31	14.14±0.87^{b}
	Hot Water	5376.34±36.51^{d}	119.05±0.80^{b}	186.94±8.91	1.44±0.73
Barks	Petroleum Ether	1346.62±17.54	8.61±2.17	27.40±0.93	0.44±0.26
	Chloroform	2112.74±21.08	24.36±1.91	119.10±8.31	3.59±0.18
	Acetone	7269.71±20.25^{b}	104.12±1.26^{c}	266.58±3.18^{c}	10.07±0.07^{c}
	Methanol	3955.48±23.38	69.51±0.96	233.02±0.61^{d}	6.92±0.40^{d}
	Hot Water	4724.97±30.93	31.29±1.54	139.92±4.05	4.54±0.05
Flowers	Petroleum Ether	216.00±32.55	32.07±4.87	23.08±0.81	1.25±0.36
	Chloroform	1106.99±74.63	22.34±1.84	96.53±1.40	2.93±0.24
	Acetone	3972.35±32.55	87.06±1.37	337.11±0.20^{b}	15.03±2.50^{b}
	Methanol	2062.11±35.56	75.98±1.12	221.20±4.05	3.17±1.30
	Hot Water	3496.48±51.96	48.91±1.75	99.92±6.59	2.47±0.91

TE – Trolox Equivalents; Fe(II)E – Fe(II) Equivalents; AAE – Ascorbic Acid Equivalents; EDTAE – EDTA Equivalents
Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$ in each column

Figure 2: Nitric oxide and Superoxide radicals scavenging activity of *T. asiatica*

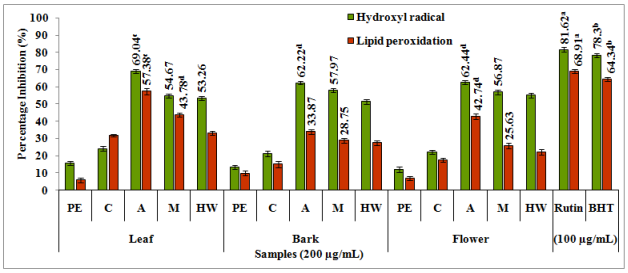


PE – Petroleum ether, C – Chloroform, A – Acetone, M – Methanol, HW – Hot water, Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$ in each assay

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid - iron EDTA. The hydroxyl radical formed by the oxidation will react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The scavenging activity of different plant parts were shown in figure 3. The OH$^{\bullet}$ scavenging activities of all the samples were investigated at the concentration of 200 µg/mL except for standards. The acetone extracts of leaves, barks and flowers showed significant levels of scavenging activities and were 69.04, 62.22 and 62.44% respectively. The methanol extracts of samples also exhibited moderate scavenging activities compared to other sample extracts.

Figure 3: Hydroxyl radical scavenging and Lipid peroxidation activity of *T. asiatica*



PE – Petroleum ether, C – Chloroform, A – Acetone, M – Methanol, HW – Hot water, Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at $p < 0.05$ where $^{a} > ^{b} > ^{c} > ^{d}$ in each assay

Lipid Peroxidation

Lipid peroxidation is an oxidative deterioration process of polyunsaturated fatty acids which is induced by radical and its inhibition results by *T. asiatica* are shown in figure 3. In this assay, *T. asiatica* extracts established strong inhibition for lipid peroxidation which was higher for acetone extract of leaves (57.38%). Other extracts, like methanol extract of leaves (43.78%) and acetone extract of flowers (42.74%) also showed comparable inhibition of lipid peroxidation at 200 µg/mL. The decreasing order of inhibition for different parts of *T. asiatica* was found to be leaves > flowers > barks.

Antibacterial activity

The leaves, barks and flowers extracts of *T. asiatica* were tested separately for their potential inhibition against pathogenic microorganisms and the results are presented in table 3. Among the different solvent extracts high antibacterial effect was shown by the considerable zone of inhibition against *S. boydii* (14 mm), *B. subtilis* (13 mm) and *S. dysenteriae* (12 mm) which were comparable to the activity of antibiotic chloramphenicol. Leaves and flowers acetone extracts also inhibited the growth of *S. dysenteriae*, *B. subtilis* and *S. boydii* with an inhibition zone ≥12 mm. Petroleum ether, methanol and hot water extracts were not as effective as acetone extracts to retard the growth of microorganisms. The gram positive *S. aureus* was found to be sensitive to none of the extracts. On the other hand, the antibiotic chloramphenicol exhibited significant inhibition against all the microorganisms tested. The acetone extracts were found to be more active against gram negative bacteria compared to gram positive bacteria.

The acetone extracts which showed positive inhibition against the bacteria *B. subtilis* and *S. boydii* were selected for the evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for active inhibition of bacterial growth (Table 4). Initially, the extracts were prepared in different concentrations such as 10, 20, 30, 40 and 50 (µg/mL) whose activities directed to study the activities of further concentrations such as 42, 44, 46 and 48 (µg/mL). From the study, it was concluded that among the acetone extracts of different parts, flowers extract possesses higher antibacterial activity against *B. subtilis and S. boydii*. It was found that among the different concentration gradients 40 – 50 µg/mL of extracts showed clear broth which depicts the active inhibition on culture growth. The lowest concentration of the extract (MIC) that will inhibit the visible growth of the microorganisms was found to be 20 µg/mL for flowers acetone extract against both *B. subtilis* and *S. boydii*. It was further optimized from the clear inhibition of bacterial growth that the MBC of acetone extract was 46 µg/mL against *B. subtilis* and 44 µg/mL against *S. boydii*. The leaves and barks acetone extracts were found to have no significant effect against both *B. subtilis and S. boydii* as compared to that of flowers.

Table 3: Antimicrobial activity of *T. asiatica* against pathogenic microorganisms

Microorganisms	Zone of inhibition (mm)															
	Leaves (1 mg/mL)					Barks (1 mg/mL)					Flowers (1 mg/mL)					Chloramphenicol (100 µg/mL)
	PE	C	A	M	HW	PE	C	A	M	HW	PE	C	A	M	HW	
K. pneumoniae	6	-	10	-	-	7	-	8	-	-	7	-	6	-	-	12
C. diphtheriae	-	-	-	-	8	-	-	-	8	-	-	-	6	8	8	10
B. subtilis	8	6	12	-	7	7	6	13	-	8	8	6	12	-	6	14
P. vulgaris	11	8	8	6	8	11	8	7	6	-	11	8	8	6	-	12
S. aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
S. typhi	-	-	-	8	8	-	-	-	8	8	-	-	-	7	8	14
S. dysenteriae	10	-	13	-	-	10	-	12	-	-	8	-	12	-	-	15
E. coli	-	-	8	-	-	-	-	8	-	-	-	-	-	8	-	14
P. aeruginosa	-	-	8	-	-	-	-	8	-	-	8	-	8	-	-	14
S. boydii	11	8	12	7	6	7	10	14	7	6	8	7	12	-	6	14

PE – Petroleum ether, C – Chloroform, A – Acetone, M – Methanol, HW – Hot water, '–' indicates no activity

Table 4: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of acetone extracts of *T. asiatica* against *B. subtilis* and *S. boydii* cultures

Microorganisms	Acetone Extracts	Concentration (µg/mL)								
		10	20	30	40	42	44	46	48	50
B. subtilis	Leaves	-	+	+	+	+	+	+	+	+
	Barks	-	+	+	+	+	+	+	+	+
	Flowers	+	++	++	++	++	++	+++	+++	+++
S. boydii	Leaves	-	+	+	+	+	+	+	+	+
	Barks	-	+	+	+	+	+	+	+	+
	Flowers	+	++	++	++	++	+++	+++	+++	+++

'–' highly turbid; '+' clear; '++' moderately clear; '+++' highly clear

DISCUSSION

Quantification of total phenolics and flavonoids

The hydroxyl groups present in the polyphenols make them good for free radical-scavenging reactions and as metal chelating agents. Moreover, the arrangement of the hydroxyl groups around the phenolic molecule is also important for antioxidant reactions [32]. The higher amount of phenolics in the acetone extracts of *T. asiatica* leaves, barks and flowers could be due to its higher solubility and it could be significantly contribute to the antioxidant capacity of that species.

Flavonoids are used as natural antioxidants in food, medicinal and nonnutritive plant materials due to their ability to inhibit and scavenge reactive oxygen species [33]. Flavonoids are potent inhibitors of molecular oxygen (O_2), and also scavenge other free radicals such as OH and NO_2 [34]. Flavonoids suppress the effects of active oxygen species (H_2O_2 and O^{2-}) in many other vulnerable biological systems [35]. Since *T. asiatica* possess good flavonoid content in leaves and barks, it could be assumed that it can have a higher free radical scavenging activity which involves the transfer of electron or hydrogen atom from flavonoids to free radicals.

In vitro Antioxidant Assays

DPPH radical scavenging activity

The DPPH assay has been widely used to analyze the antioxidant activity of plant extracts and foods as free radical scavengers or hydrogen donors [36]. The antiradical scavenging activity of different extracts of *T. asiatica* would be related to the nature of phenolics, flavonoids etc. which contributes to their electron transfer or hydrogen donating ability [37]. The significant activity of acetone extract of leaves may be due to the presence of phenolic compounds in the plant parts. On the other hand, the DPPH radical scavenging efficiency of extracts from *T. asiatica* might have also been partly attributed to Millard reaction products other than phenolic constituents because they also effectively participate as radical scavengers [38]. The higher apparent antioxidant capacity of smaller molecules due to their better access to the DPPH radical site suggest that the extracts may contain more smaller compounds than larger compounds [39].

ABTS radical cation scavenging activity

The ABTS radical is soluble in both aqueous and organic solvents, is not affected by ionic strength and can be used to measure the antioxidant capacity of hydrophilic and lipophilic compounds in test samples [40]. Moreover, the radical is suitable for evaluating antioxidant capacity of phenolics due to their comparatively lower redox potentials (0.68 V). Many phenolic compounds can thus react with the ABTS radical because of this thermodynamic property [41]. Apart from these, Hagerman *et al*. have reported that the high molecular weight phenolics have more ability to quench free radicals ($ABTS^{\bullet+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific groups [42]. The ABTS assay indicated that the extracts of *T. asiatica* possess strong hydrogen donating ability and could serve as free radical scavengers by acting as primary antioxidants when they are ingested along with nutrients.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay is used to measure the antioxidant effect of any substance in the reaction medium as its reducing ability. Yen and Duh reported that the reducing power of bioactive compounds, mainly low and high molecular phenolics, was associated with antioxidant activity, specifically scavenging of free radicals [43]. It has been also proved that the potential antioxidants through *in vitro* ferric-reducing antioxidant power assay increased the total antioxidant capacity of blood plasma [44]. Thus the ferric reducing power of different extracts of *T. asiatica* reveals that there are compounds in the acetone extracts which have high affinity to the ferrous ions and thereby quench/scavenge them through redox reactions.

Phosphomolybdenum reduction assay

Phosphomolybdenum assay is mainly based on the ability of plant extracts to reduce Mo(VI) to Mo(V) and subsequent formation of green phosphate/Mo(V) complex at an acidic pH. Being simple and independent of other antioxidant measurements commonly employed, the application of assay was extended to plant extracts [24]. Since the antioxidant activity is expressed as the number of equivalents of ascorbic acid, the total antioxidant capacity observed for the extracts of *T. asiatica* can be correlated with its free radical

scavenging activity. The reduction of Mo (VI) to Mo (V) by the leaves, barks and flowers extracts of *T. asiatica* may be due to the electron transfer or hydrogen ion transfer by the bioactive compounds, specifically phenolics and flavonoids present in the respective plant parts.

Metal chelating activity

Iron can undergo Fenton reaction and produces reduced metals which may form highly harmful hydroxyl radicals and thereby contributing to oxidative stress [45]. Reports reveals that chelating agents which form σ- bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [46]. Moreover, antioxidants inhibit interaction between metal and lipid through formation of insoluble metal complexes with ferrous ion. Therefore, the metal chelating assay reveals that acetone extract for all the parts have shown effective activity, suggesting that its action as antioxidant may be related to its sequestering of Fe^{2+} ions that may otherwise catalyze Fenton type reactions or participate in metal catalyzed hydroperoxide decomposition reactions.

Nitric oxide radical scavenging assay

Reactive nitrogen species, formed during their reaction with oxygen or with superoxides are very reactive and can be implicated for inflammation, cancer, and other pathological conditions [47]. These compounds are responsible for altering the structural and functional behavior of many cellular components. The phytochemicals possess the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health. Since, the acetone extracts of leaves and barks of *T. asiatica* showed higher scavenging activity, it is clear that it can be used for reducing the deleterious effects caused by the reactive nitrogen species in human body.

Superoxide radical scavenging activity

Superoxide radical acts as a precursor of more reactive oxygen species like hydrogen peroxide, hydroxyl and singlet oxygen and is known to be a very harmful species to cellular components [48]. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potent precursors of highly reactive species such as hydroxyl radical and thus the study of scavenging of this radical is important [49]. Since, the acetone extract of leaves and barks of *T. asiatica* showed appreciable percentage of scavenging activity against superoxide radical, it can be used against adverse effects caused by superoxide radical in the body. The active principles in the plant extracts may eliminate the radical by its reduction to attain the octant stage or through the formation of water molecule.

Hydroxyl radical scavenging activity

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules like lipids, polypeptides, proteins and DNA, especially thiamine and guanosine [50]. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxyl cyclohexadienyl radical. The resulting radical can undergo further reactions, such as reactions with oxygen to give peroxyl radical, or decompose to phenoxyl type radicals by water elimination [48]. Hagerman *et al*. have also reported that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by tannins than their specific functional groups [42]. Thus, the hydroxyl radical scavenging of *T. asiatica* acetone extracts can be directly related to the amount of phenolics and tannins present in the sample.

Lipid peroxidation assay

In the food processing industries, flavonoids have been shown to inhibit heat or chemical initiated lipid peroxidation as well as chelating metallic and super oxide ions [33]. The phytoconstituents in the plant extracts can reduce the highly reactive and transient peroxyl radicals that are involved in lipid peroxidation [51]. There was a significant chelating capacity as the extracts reduced the concentration of the catalyzing transition metal in lipid peroxidation [52]. The active components in the acetone extracts of leaves, barks and flowers can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants and therefore inhibit lipid peroxidation [53].

Antibacterial Activity

T. asiatica is a magnificent medicinal plant characterized by its significant antimicrobial activity against the tested pathogens. The different solvent extracts of leaves, barks and flowers of *T. asiatica* exhibited different range of inhibition where acetone extracts showed high degree of antibacterial activity against the selected organisms. It is well noted that chloroform and methanol extracts showed less activity than acetone extracts. This may be due to the same active substances were present in chloroform/methanol extracts in low concentrations or active substances were soluble in acetone and not present in chloroform and methanol extracts.

The earlier reports state the antibacterial activity of *T. asiatica* at the range of 8 – 14 mm of inhibition against most of the tested organisms like *P. vulgaris*, *B. subtilis* and *S. boydii* by all the sample extracts. The current observation has been deviated from other studies by the difference in the concentrations of the solvent and samples tested. It highlights the use of acetone extract for potential inhibition against most of the tested pathogens and in specific the MIC and MBC of their crude extract concentration was identified up to 46 µL of aliquot volume. Similar antibacterial activity against different bacterial pathogens was reported for few Rubiaceae members by Jayasinghe *et al*. [14] and Usha *et al*. [54] whereas for leaves extracts of *T. asiatica* by Choudhury *et al*. [55]. The earlier studies also reported the antibacterial activity of few species of Rubiaceae members against *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas aeruginosa.* The ethanol soluble fraction of hydroalcoholic extract of *Psychotria reevesii* aerial parts exhibited an inhibition of 15.7 mm against *Staphylococcus aureus* [6]. It is evident that the extracts used in the study was highlighted in terms of concentration is considerably lower amount tested produced higher zone of inhibition by plant extracts.

B. subtilis produces the enzyme subtilisn, which has been reported to cause dermal allergic or hypersensitivity reactions in individuals repeatedly exposed to this enzyme in industrial settings. The gram negative bacteria, *E. aerogenes* are a nosocomical and pathogenic bacterium that causes opportunistic infections [56]. *K. pneumoniae* is an important cause of human infections and several diseases viz., urinary tract infections, noscomial infections, pneumonia, septicemias and soft tissue infections. The diseases caused by *K. pneumoniae* can result in death of patients who are immunodeficient. Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections and neonatal meningitis. In rare cases, virulent strains are also responsible for haemolytic uremic syndrome, peritonitis, mastitis, septicaemia and gram negative pneumonia [57]. *B. cereus* is responsible for a minority of food borne illnesses (2-5%), causing severe nausea, vomiting and diarrhea [58]. The antibacterial findings suggest that the plant extract can be effectively used to treat these infectious disease caused by these different bacteria.

Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidences of their inherent ability to modify the body's reaction to allergen, bacteria, virus and carcinogens. Moreover, they exhibit antiallergic, anti-inflammatory, antimicrobial and anticancer activity. The high molecular weight phenolics are also known to possess general antimicrobial and antioxidant activities [59]. Therefore, it can be assumed that the phenolics, flavonoids and tannins present in the plant extracts may contribute to the activity.

CONCLUSIONS

The study concluded that *T. asiatica* possesses significant phenolic and flavonoid contents which contribute to the pronounced antioxidant and antimicrobial activities. The extracts have great potential as antimicrobial compounds, especially in the treatment of

infectious diseases caused by resistant microorganisms. However, it has to be considered that the results of this work can be used as a lead to continue the search of active substances in the extracts for the development of indigenous botanical resources.

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