ANTIMICROBIAL AND FREE RADICAL SCAVENGING ACTIVITY OF LEAF AND STEM EXTRACT OF LIMONIA ALATA WIGHT AND ARN.

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INTRODUCTION

Medicinal plants have long been utilized as a source of therapeutic agents worldwide. Higher plants have been used for centuries as remedies for human diseases [1]. Plants are known to produce certain chemicals which are naturally toxic to bacteria [2]. This has encouraged research into a screening of plants for antibacterial and antifungal activities [3]. Free radicals are the character of reactive oxygen species (ROS), which contain all highly reactive, oxygen-containing molecules. 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 of responding with layer lipids, nucleic acids, proteins and chemicals and other little particles, ensuing in cell damage [4]. ROS, sometimes denominated as actives oxygen species, are forms of activated oxygen, which include free radicals such as superoxide ions and hydroxyl radicals (OH) [5].

Living organisms have antioxidant defense 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]. The most active path to remove and diminish the action of free radicals which produce the oxidative stress is antioxidative defense technique. Antioxidants are those substances which dominate free radical chain reaction breaking quality. Recently, there has been a promote of benefit in the therapeutic ability medicinal plants as antioxidants in reducing oxidative stress-induced tissue damage [9].

Many plants contain cancer prevention agent mixes, and these mixes secure cells against the harming impacts of receptive oxygen species (ROS, for example, singlet oxygen, superoxide, peroxyl radicals, OH, and peroxyxinirite) which brings about oxidative pressure prompting cell harm. Epidemiological examinations have shown the connection between the plant antioxidant and lessening of unending infections [10-12]. Oxidative stress is involved in the pathology of cancer, arteriosclerosis, malaria and rheumatoid arthritis, and could play a role in neurodegenerative diseases and aging processes [15].

Pediatus tithymaloides (L.) Poit., belonging to Euphorbiaceae, is an erect shrub originating from tropical America. Indian folklore uses P. tithymaloides as antiviral, antibacterial, antihemorrhagic, antitumor, abortive, anticancer, and anti-inflammatory agent [16].

The high cost of natural antioxidants has led to the use of synthetic antioxidants. However, studies conducted subsequently have demonstrated that synthetic antioxidants have toxic effects and, consequently, restrictions have been imposed on their use. Therefore, researchers have focused their studies on plant-derived natural antioxidants [6]. Antioxidant are also defined as a substance which are capable of inhibiting a specific oxidizing enzymes or a substance that reacts with oxidizing agents before causing damage to other molecules.

ABSTRACT

Objective: In the present study, antioxidant activities leaves and stem of the petroleum, chloroform, acetone, and methanolic extracts from Limonia alata (L.). Leaf and stem were investigated by employing established in vitro studies. The leaves and bark are used for the fomentation of rheumatic pain; the dried fruit is useful in malignant and pestilent fevers and is used as an antidote for poisons, the folklore claim suggests that the leaf is showing wound healing property. L. alata is belonging to the family Rutaceae.

Methods: The ability of the plant extract to act as hydrogen/electrons donor or scavenger of radicals was determined by in vitro antioxidant assays using 2,2-diphenyl-1-picryl-hydrazyl free radical (DPPH) scavenging, reducing power assay, superoxide radical (O2−) scavenging activity, phosphomolybdenum assay, ferric reducing antioxidant power and metal chelating activity, was performed to study the antioxidant potential and antimicrobial activities of the plant extract of leaves and stem of L. alata.

Result: Results are evaluated higher in leaves, stem and root of L. alata (L.) recorded total phenol (59.95±5.30). The present state of work was designed to evaluate the phytochemical, antioxidant as well as to screen the antimicrobial present in the plant extracts of L. alata. The plant L. alata petroleum ether extract of leaf showed greater IC50 for DPPH assay (154.8 µg/mL) and compare to another extract, higher reducing power activity stem in methanol (0.99EDTAE/100 g extract), better phosphomolybdenum reduction (0.512 mg/g extract) higher ferric reducing power (11542.3MmolFe(II)E/mg) extract and higher superoxide radical scavenging activity in leaf extract (332.13%). However, the better metal chelating ability was shown by the water extracts of the leaf (18.24 EDTAE/100 g) compared to other solvent extracts.

Conclusion: The result indicates the antioxidant and antibacterial activity potential of L. alata.

Keywords: Limonia alata, 2,2-diphenyl-1-picryl-hydrazyl free radical assay, Reducing antioxidant power, Antioxidant activity and antimicrobial activity.

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or a substance that sequesters metal ions or even a substance capable of repairing system such as iron transporting protein [2]. As such, production of free radicals and other ROS in the human body by numerous physiological and biochemical processes is reported [8].

METHODS

Plant material

The leaf, stem and root parts of L. alata were collected from Maruthamalai 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/5723/2016/Tech.,11180) and the voucher specimen has been deposited in Bhannarath University Herbarium, Department of Botany, and Coimbatore. The collected fresh plant for leaves, stem, and root was cleaned thoroughly with running tap water to remove dust and shade dried for a week at room temperature. The powers were in the airtight container.

Plant extracts preparation

The powdered plant material was extracted as one of the extraction method of Soxhlet extractor based extracted continuously with petroleum ether, chloroform, acetone, and methanol. Each time before extracting with the following dissolvable, the thimble was dried in hot air stove underneath 40°C. The distinctive dissolvable concentrates were thought by rotating vacuum evaporator and after that air dried. The dried concentrate got with every dissolvable was weighed. The rate yield was communicated as far as air-dried weight of plant material.

Quantification of total phenolics, tannins, and flavonoids

Quantification of total phenolics and tannin

The total phenol content was determined according to the method described by Makkar (2003). 100 µL aliquots for plants extracts (5 mg/mL) were taken in the test tubes and made up to the volume of 1 mL with distilled water. Then, 500 µL of Folin-Ciocalteu reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added basically in each tube. Immediately vortexing the reaction mixture, then the test tubes were placed in the dark room for 40 min and after the absorbance was 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.

Using the same extract the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP) Makkar (2003) 75 mg of PVPP was weighed into a 2 mL Eppendorf tube and to this 900 µL distilled water, and then 750 µL of the sample extracts were added. The content was vortexed and kept in the test tube at 4°C for 4 h. Then the sample was centrifuged at 4000×g for 10 min at room temperature and the supernatant was collected. This supernatant has just basic phenolics other than the tannin substance of the example was taken as the substance of non-tannin phenolics. From the above results, the tannin substance of the example was figured as follows after:

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

Quantification of total flavonoids

The flavonoid content of the considerable number of concentrations was evaluated as itgoes about as a noteworthy antioxidant in plants diminish oxidative anxiety. Assessed according to portrayed by Zhishen et al. (1999). Initially, 150 µL of all the plant extract were taken in different test tubes. To each extracts 2 mL of distilled water was added. Then 150 µL of NaNO2 was added to all the test tubes followed by incubation at room temperature for 6 min. After incubation 150 µL of AlCl3 (10%) was added to all the test tubes. The test tubes were incubated for 6 min 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 min at room temperature. The pink color developed due to the appearance of flavonoids was read spectrophotometrically at 510 nm. The amount of flavonoids was calculated as rutin equivalents.

In vitro antioxidant studies

2,2-diphenyl-2-picrylhydrazyl free radical (DPPH) radical scavenging activity (Shimada et al., 1992)

The antioxidant movement of the concentrates was resolved regarding hydrogen giving or radical searching capacity, utilizing the steady radical DPPH, as indicated by the strategy for Blois (1950) leaf and stem sample extracts at various concentrations (20-100 µL) was added to 5 mL of 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured using the spectrophotometer at 517 nm. Methanol was filled in as blank and arrangement without remove filled in as a control. The mixture of methanol, DPPH and standard (ascorbic acid) filled in as a positive control. Radical scavenging activity was communicated as the restraint percentage of free radical by the sample was figured utilizing the formula. More significantly the IC50 of the extracts were also calculated.

Assay of superoxide radical (O2−) scavenging activity

The examine was found in the capacity of the example concentrate to check development by searching superoxide radicals begin in riboflavin light nitroblue tetrazolium (NBT) framework (Beauchamp and Fridovich, 1971). Every 3 mL response blend contained 50 mM sodium phosphate buffer (pH 7.6). 2.33 µg riboflavin and 12 mM EDTA, and 11.55 g NBT. Response was begun by lighting up the response blend with of test extracts (100 µL) for 90 s. Response blend with extricate kept in dull filled in as a negative control while the blend without separate was taken as clear. Promptly after brightening, the absorbance was measured at 590 nm. The action was contrasted with ascorbic acid. The rate lessening of superoxide anion creation was figured utilizing the accompanying equation:

Level of restraint=([Control OD-Sample OD]/Control OD)×100

Reducing power assay

The reducing power of different solvent extracts of L. alata was determined by the method reported by Oyaizu, 1986. 500 µL of the extract was taken in 2.5 mL of 0.2 M 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°C for 20 min. After the incubation, 2.5 mL of 10% trichloroacetic acid was added. The content was centrifuged at 3000 rpm for 10 min. 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.

Percentage reducing power=([Control OD-Sample OD]/Control OD)×100

Phosphomolybdenum assay

The antioxidant capacity of the extracts has been determine with the phosphomolybdenum reduction assay present to Prieto et al. (1999). The assay was based on the reduction of the extract and subsequent formation of a complex (green color). 0.5 mL of extract combined with

[Image: Fig. 1: DPPH scavenging activity of L. alata leaf and stem extract]
3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was incubated at 95°C for 90 min. The absorbance was taken at 695 nm using a spectrophotometer. The results were calculated in ascorbic acid equivalents.

Percentage of phosphomolybdenum=(Control OD-Sample OD/Control OD)×100

Metal chelating activity

Principle

Iron II chelating activity was calculated by the check of the creation of iron(II)-ferrozine complex following preincubation of the sample. The Fe²⁺ was monitored by measuring the formation of ferrous iron-ferrozine complex against methanol blank at 562 nm. The chelating of ferrous ions by various extracts in the plant was estimated by the method of (Dinis et al., 1994).

The chelating of ferrous ions by various extracts of L. alata was calculated. Initially, about 100 µL of the extract sample was added to 50 µL of 2 mM FeCl₃ solution. Then the reaction was initiated by the addition of 200 µL of 5 mM ferrozine and the test tubes were vortexed well and left standing at room temperature for 10 min. The reaction mixture containing deionized water in place of sample was considered as the negative control absorption of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). EDTA was against the standard metal chelating specialist, and the outcomes were communicated as mg EDTA counterparts/extricate chelate the ferrous particle was computed by:

Rate chelation=(1-\[ABS test/ABS control\])×100

Ferric reducing antioxidant power (FRAP)

The antioxidant contents of phenolic extracts of samples were considered according to the procedure explained by Pulido et al. (2000). FRAP reagent (2.7 mL), made freshly and incubated at 37°C, it was mixed with 270 µL of distilled water and 50 µL of extract or methanol (for the reagent blank). The test sample and reagent clear were brooded at 37°C for 30 min in a water shower. The last weakening of the test in the response blend was 1/34. The FRAP reagent making 2.5 mL of 20 mM/L TPTZ (2,4,6-tripyridyl-s-triazine) arrangement in 40 mM/L HCl in addition to 2.5 mL of 20 mM/L FeCl₃·6H₂O and 25 mL of 0.3 M/L acetic acid derivation support (pH 3.6) clarified by Siddhuraju and Becker, (2003). Toward the finish of hatching, the absorbance readings were taken quickly at 593 nm. Results were ascertain in ferrous sulfate counterparts.

Antibacterial activity

The microbiological assay of the ethanol extract of L. alata leaf was done by comparing the inhibition of the growth by measured concentration of the antibiotics. The bacterial strains used were responsible for producing skin diseases and diarrhea to humans. The bacterial strains used were Streptococcus aureus, and Escherichia coli, and then the fungal culture of Candida albicans, and Fusarium oxysporum were obtained from T. STANES and Company Phyto-pharma testing lab. All these cultures were maintained on nutrient agar plates at 4°C, respectively in lab.

Anti-fungal activity

Potato dextrose medium was making and transferred into the incubated Petri dish. 200 µL of the standardized fungal inoculum was spread on agar medium using a sterile cotton swab. The 40 µL extracts were added to the well on the agar medium. Amikacin (AK²⁺) (10 µg/disc) was used as reference standard to determine the sensitivity of each microbial species tested. All the Petri plates were incubated at 27°C for 72 h. After the incubation period, the diameter of the zone of inhibition was measured (Table 2). C(sa)=concentration of sample C(st)=concentration of standard A(st)=area peak in sample A(sa)=area of peak in standard.

Statistical analysis

All analyses were performed 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±standard deviation used Excel 2003.

RESULTS AND DISCUSSION

Quantification of total phenolics, flavonoid and tannin

The results of total phenolics and tannin contents are showed in Table 3. When obtained for methanol extract of L. alata leaves revealed highest phenolic in the methanolic extract (33.87 g/100) and stem in tannin chloroform (2.65 g/100) extract respectively. Then, the stem of L. alata revealed highest phenolic in methanolic (33.03 g/100) g and tannin in chloroform (2.26/100) g extract. The results of flavonoid content are presented in Table 3. In this estimation, in the methanol extract of L. alata stem revealed maximum amount of flavonoid content (38.6 g/100) followed by methanol extract of the leaf (38.6±2.03/g).

Antioxidant activity

Radical scavenging activity using DPPH method

In L. alata leaf extract in chloroform extract shows (154.8 µg/mL) the higher inhibitory activity whereas methanol shows the minimum (12.33 µg/mL) inhibitory activity. In stem petroleum ether extract shows (132.9 µg/mL) the higher inhibitory activity whereas methanol shows (26.33 µg/mL) the minimum inhibitory activity. The inhibitory percentage of standard natural antioxidant-rutin and synthetic antioxidant-butylated hydroxytoluene was found to be much better than that of plant extracts. Since the DPPH assay can accommodate many samples in a short period and sensitive enough to detect active ingredient at low concentration, the DPPH radical has been widely used to test the capability of mixture as free radical scavengers or hydrogen donors to evaluate the antioxidant activity of the plant extracts and foods (Soares et al., 1997).

Phosphomolybdenum assay

Phosphomolybdenum assay is successfully used to determine the ability of extracts to reduce Mo (VI) to Mo (V) and subsequent formation of

Table 1: Composition of nutrient agar medium for bacterial culture

| S. No | Composition | Quantity |
|-------|-------------|----------|
| 1     | Peptone (g) | 5        |
| 2     | Beef extract (g) | 3        |
| 3     | Sodium chloride (g) | 5        |
| 4     | Agar (g) | 15       |
| 5     | Distilled water (mL) | 1000     |
| 6     | pH | 7        |

Table 2: Composition of nutrient agar medium for bacterial culture

| S. No | Composition | Quantity |
|-------|-------------|----------|
| 1     | Potato (g) | 200      |
| 2     | Dextrose (g) | 20       |
| 3     | Agar (g) | 15       |
| 5     | Distilled water (mL) | 1000     |
| 6     | pH | 6.5       |
Table 3: Total phenolics, tannins and flavonoid content of leaf and stem extract of *L. alata*

| S. No | Plant material | Extract | Total phenol (GAE mg/100 g) | Tannin (GAE mg/100 g) | Flavonoid (RE mg/100 g) |
|-------|----------------|---------|-----------------------------|----------------------|-------------------------|
| 1     | L.A. Leaves    | Pet ether | 20.6±1.80                  | 0.6±2.18             | 28.1±8.74               |
| 2     | L.A. Leaves    | Chloroform | 27.2±0.55                  | 2.26±3.10*          | 25.6±3.51               |
| 3     | L.A. Leaves    | Acetone   | 20.38±1.02                 | 0.45±0.26            | 23.5±1.20               |
| 4     | L.A. Leaves    | Methanol  | 33.87±0.58                 | 0.01±1.05            | 38.6±2.03               |
| 1     | L.A. Stem      | Petroleum | 27.5±1.80                  | 0.94±2.18            | 19.7±6.74               |
| 2     | L.A. Stem      | Chloroform | 32.94±0.55                 | 2.65±3.12*          | 38.9±3.51               |
| 3     | L.A. Stem      | Acetone   | 20.87±1.02                 | 0.5±0.26             | 26.3±1.20               |
| 4     | L.A. Stem      | Methanol  | 33.03±0.58                 | 0.14±1.05            | 45.4±2.03               |

Values are mean of replicate determination (n=3)±SD. GAE: Gallic acid equivalence, RE: Rutin equivalence, SD: Standard deviation. *L. alata: Limonia alata*

green phosphate/Mo(V) complex at an acid pH. The total antioxidant capacity of different solvent extracts of leaf, stem of *L. alata* was analyzed and shown in Fig. 2. In *L. alata* leaf showed higher activity in most of its solvents compared to the extracts of the stem. The methanol extract of leaf and stem (0.512 mg/g; 0.4212 mg/g extract) has highest phosphomolybdenum reduction compared to other solvent extracts.

**FRAP assay**
The ferric decrease antioxidant power assay determines the antioxidant result of any substance in the reaction medium as reducing ability. Antioxidant potential of leaf and stem extract of *L. alata* were estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) and the results are given in Fig. 3. *L. alata* methanol extract of leaf and stem methanol extract from stem revealed higher activity and the values were 32.14.1MmolFe(II)/E/mg and 11542.3MmolFe(II)E/mg extract. The FRAP test measures the antioxidant impact of any substances in the response medium as diminishing capacity.

**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 *L. alata* was calculated and the results were presented in Figs. 4 and 5. The *L. alata* methanol extract in stem (0.99 EDTAE/100 g) and leaf (0.68 EDTAE/100 g) in methanol extract shows higher reducing power compared to other extracts; the values were 9.97 EDTAE/100 g extract. The values were lower for chloroform extract.

**Superoxide radical (O2−•) scavenging activity**
The superoxide anion radical scavenging activities of *L. alata* leaf and stem are shown in Fig. 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 *L. alata* leaf and stem showed higher superoxide radical scavenging activity (332.13% and 302.15%) compared to other solvent extracts at a concentration of 100 μg/mL. Superoxide radical is essential because it conducts as the precursor for other major ROS such as hydrogen peroxide, hydroxyl and singlet oxygen (Lee et al., 2004). The potential technique of scavenging of superoxide anion radicals in *L. alata* can be due to the effective principles in the plant extracts which may reject the superoxide anion radicals which are caused through the photo-illumination process.

**Metal chelating activity**
Ferrozine can quantitatively chelate with Fe2+ and shape a red shaded complex. This response is constrained within sight of other chelating operators and results in a diminishing of the red shade of the ferrozine-Fe2+ complex. Estimation of the shading lessening gauges the chelating movement to contend with ferrozine for the ferrous particles (Soler-Rivas et al., 2000). The antioxidants present in plant extract forms a coordinate complex with the metal ions (chelating activity) and inhibit the transfer of electrons. This oxidation reaction is arrested, and no free radicals are produced. The Fe2+ chelating capacity of different solvent extracts of *L. alata* leaf and stem are shown in Fig. 7. In *L. alata* maximum chelation’s were observed for the methanol extract of leaf and stem (18.24 EDTAE/100g; 20.156 EDTAE/100g) extract.

**Antimicrobial activity**
The methanolic extract of *L. alata* leaf extracts was checked for their antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans*. The inhibition of microbial growth was observed. The zone of inhibition was found and measured and is presented in Table 4. The images were presented in the Plates 1-4. Among the two bacteria and two fungal zone of inhibition is higher in *E. coli* of 31 mm and in 27 mg/ml of *L. alata* methanol extract.
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alata leaf extracts. The anti-bacterial activities are against *S. aureus* significantly higher in *E. coli*. Then, fungal zone of inhibition is higher in *C. albicans* of 27 mm and 24 mg/mL of *Ziziphus oenoplia* leaf extracts. The anti-fungal activities are against *F. oxysporum* significantly higher in *C. albicans*. In case bacterial activities are against *Staphylococcus S. aureus* is significantly lower than antibacterial. Hence, the plant has higher antibacterial activity it can be used as good medicine.

Table 4: Antimicrobial activity of methanolic leaf extract of *L. alata*

| Name of the pathogen | Test parameter | Zone of inhibition in mm | Antibiotic concentration (10 mg/mL) | LLM extract concentration (10 mg/mL) |
|----------------------|----------------|--------------------------|-----------------------------------|------------------------------------|
| *S. aureus*          | Anti-bacterial | 28 mm                    |                                    | 18                                 |
| *E. coli*            |                | 31 mm                    |                                    | 14                                 |
| *F. oxysporum*       | Anti-fungal    | 14 mm                    |                                    | 21                                 |
| *C. albicans*        |                | 27 mm                    |                                    | 12                                 |

L. alata: Limonia alata, *S. aureus*: Streptococcus aureus, *E. coli*: Escherichia coli, *F. oxysporum*: Fusarium oxysporum, *C. albicans*: Candida albicans, LLM: Limonia alata leaf methanolic extract

**CONCLUSION**

*L. alata* leaf can be valuable natural antioxidants, antimicrobial activity source which seemed to provide potential nutraceutical for human health. Further, detailed exploration chemical studies and screening for medicinal properties with provide a cost effective and reliable source of medicine for the welfare of humanity.

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Plate 3: Fusarium oxysporum

Plate 4: Candida albicans

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