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Phytochemical composition and *in vitro* antioxidant activity of aqueous extract of *Aerva lanata* (L.) Juss. ex Schult. Stem (Amaranthaceae)

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ABSTRACT

Objective: To analyze the phytochemical composition and *in vitro* antioxidant properties of aqueous extract of *Aerva lanata* (*A. lanata*) stem. **Methods:** During the preliminary phytochemical analysis, the aqueous extract of *A. lanata* was screened for the presence of carbohydrates, proteins, phenolic compounds, oil and fats, saponins, flavonoids, alkaloids, tannins and phytosterols. Antioxidant activity of the extract was determined by 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity, metal chelating activity, reducing power activity and DNA damage inhibition activity. Analysis of phenolic compounds was performed by Folin-Ciocalteau reagent method and gradient high performance liquid chromatography technique. **Results:** Preliminary phytochemical analysis exhibited the presence of phenolic compounds, saponins, flavonoids, tannins and phytosterols as major phytochemical groups. The extract exhibited high 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity (IC_{50}= 110.74 μg/mL), metal chelating activity (IC_{50}= 758.17 μg/mL), reducing power activity and DNA damage inhibition efficiency. The extract was reported to possess a high amount of total phenolic content and some of them were identified as gallic acid (3,4,5-OH), apigenin-7-*O*-glucoside (apigetrin), quercetin-3-*O*-rutinoside (rutin) and myricetin (3,5,7,3,4,5-OH) by high performance liquid chromatography analysis. The extract was found non toxic towards human erythrocytes in the hemolytic assay (IC_{50}= 24.89 mg/mL). **Conclusions:** These results conclud that *A. lanata* stem possesses high antioxidant activity and can be used for the development of natural and safe antioxidant compounds.

1. Introduction

Free radicals are the molecules with unpaired electrons and commonly called reactive oxygen species (ROS). Free radicals are generated during the process of cellular oxidation, some examples includes superoxide anion, hydrogen peroxide, hydroxyl and nitric oxide radical. These radicals are electrically charged, unstable and highly reactive in nature. It reacts with nucleic acids, mitochondria, proteins and enzymes and resulted in their damage in the cell. However, antioxidant defense system protects the cell from the free radical mediated oxidative stress. When there is overproduction of free radicals or the failure of an antioxidant defense system, these radicals resulted in tissue injury and cause numerous physiological disorders in the body *viz*, cancer, Parkinson's disease, Alzheimer's disease, myocardial infarction and diabetes[1-6]. It has been proved that a diet rich in antioxidants strengthens the antioxidant defense system and can effectively neutralize the free radicals in body. Several synthetic compounds such as butylhydroxyanisole and butylhydroxytoluene are extensively used as antioxidants in food industries. Scientific investigations reported that the high doses and/or long-term exposure to these compounds can cause toxic symptoms and tumors in animal[7]. It is reported that the consumption of certain fruits and vegetables reduces the chances of various diseases in humans. This is often attributed to the phytochemicals present within these fruits and vegetables plants[8,9]. The medicinal plants which contain the high

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amount of polyphenols are considered to be good source of natural antioxidant compounds and more often possess higher antioxidant potential than that of dietary fruits and vegetables. Consumption of these plant products certainly prevents the free radical mediated damage in the cell and therefore protects the body from several health problems[10-12]. These antioxidant compounds can be used as natural antioxidant additives or nutritional supplements in the food products. As of natural origin, these antioxidants are much safe to use. Thus, much attention has been focused on the investigation of natural antioxidant compounds from plants, which can effectively scavenge ROS.

Aerua lanata (L.) Juss. ex Schult. (*A. lanata*) belongs to the family Amaranthaceae and is a tropical plant and growing in India, Arabia, Africa, Sri Lanka, Philippines and Java. *A. lanata* is a weed that is distributed widely in India, especially in warm and plane areas. *A. lanata* is extensively used in traditional medicinal systems to cure a variety of disorders such as helminthic infection, diabetes, inflammation, skin diseases, kidney stone, headache, cough, cholera, dysentery and diarrhea. In the last two decades *A. lanata* have been extensively studied for its medicinal properties by using advanced scientific techniques and reported to possess diuretic activity[13], urolithiasis activity[14], anti-inflammatory activity[15], antimicrobial activity[16], anti-diabetic activity[17], nephroprotective activity[18] hepatoprotective activity[19,20], anthelmintic activity[21] and antidiarrhoeal activity[22]. Systematic investigation of this plant extracts for its medicinal properties could provide an important input to pharmaceutic industry.

In this study we aim to investigate the aqueous extract of *A. lanata* stem for its phytochemical composition, DPPH radical scavenging activity, metal chelating activity, reducing power activity, DNA damage inhibition efficiency, concentration of phenolic compounds and hemolytic activity by various *in vitro* methods.

2. Materials and methods

2.1. Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Sodium carbonate (Na_2CO_3) was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Methanol (HPLC grade), ferrozine, ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, ascorbic acid, hydrogen peroxide (H_2O_2), sodium chloride (NaCl), Folin-Ciocalteau reagent and gallic acid were purchased from SRL Pvt. Ltd. (Mumbai, India). Plasmid DNA kit containing pBR322 plasmid DNA, agarose gel, ethidium bromide, Tris borate EDTA buffer (TBE buffer) was purchased from Medox Biotech India Pvt. Ltd. (Chennai, India). All other chemicals used were of analytical grade.

2.2. Plant material

A. lanata was collected from the natural population growing in the Seshachalam Forest Area, Chittoor district, Andhra Pradesh, India, during December 2009. The plant material was carried to the Molecular and Microbiology Research Laboratory, VIT University. A voucher specimen was maintained in our laboratory for future reference (AL/VIT/MMRL/30.12.2009-1).

2.3. Processing of plant

The stem of *A. lanata* was collected and washed thoroughly in distilled water and cut into small pieces. The stem was shade dried at room temperature. Dried stem fragments were consistently ground using the mechanical grinder to make delicate powder. The powder was extracted in distilled water using a Soxhlet apparatus. These extracts were concentrated with a rotary evaporator and dried using lyophilizer. Dried extract was collected in an air tight receptacle and stored at 4 ℃ up for further use.

2.4. Phytochemical screening

Phytochemical screening of the stem of *A. lanata* was carried out by using the standard protocols[23]. The stem extract was screened for the presence of phenolic compounds, saponins, flavonoids, tannins, proteins, carbohydrates, alkaloids, oil and fats and phytosterols.

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of aqueous extracts of *A. lanata* stem was performed by using standard protocols reported earlier[24]. The extract was diluted in methanol to make 10, 20, 40, 60, 80 and 100 μg/mL dilutions. Two milliliters of each dilution were mixed with 1 mL of DPPH solution (0.2 mM/mL in methanol) and mixed thoroughly. The mixture was incubated in the dark at 20 ℃ for 40 min. Absorbance was measured at 517 nm using UV-Vis spectrophotometer with methanol as blank. Each experiment was performed in triplicates at each concentration.

The percentage scavenging of DPPH by the extracts was calculated according to the following formula:

Percentage DPPH radical scavenging=[(Ac − At) / Ac] × 100

Here.

Ac is the absorbance of the control (DPPH); At is the absorbance of test sample.

2.5.2. Metal chelating activity

Metal chelating activity of aqueous extracts of *A. lanata* stem was performed according to the standard protocols

reported earlier[25] with some modification. 2 mL of crude extract (125, 250, 500 and 1 000 μg/mL) was mixed with 100 μL of 2 mM $FeCl_2$ and 400 μL of 5 mM ferrozine solutions and allowed to react for 10 minutes at room temperature. The absorbance at 562 nm of the resulting solutions were measured and recorded. Mixture of $FeCl_2$ and ferrozine was used as control. Each experiment was performed in triplicates at each concentration.

The percentage inhibition of the ferrous ion was calculated by comparing the results of the test with those of the control using the formula.

Percentage metal chelating activity =[1 − (At / Ac)] × 100

Here, At is the absorbance of test sample. Ac is the absorbance of the control.

2.5.3. Reducing power activity

The reducing power of aqueous extract of *A. lanata* stem was determined by ferric reducing power assay[26]. Plant extract (1 mL) at different concentrations (125, 250, 500 and 1 000 μg/mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1 % $K_3Fe(CN)_6$. The mixture was incubated at 50 ℃ for 20 min. A volume of 2.5 mL of Trichloroacetic acid (10%) was added to the mixture, and centrifuged at 3000 rpm for 10 min in a cooling centrifuge. 2.5 mL of the supernatant was mixed with equal volume of distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance was measured at 700 nm using a UV-Visible spectrophotometer. Ascorbic acid was used as positive control. Higher absorbance of the reaction mixture indicated greater reductive potential. Each experiment was performed in triplicates at each concentration.

2.5.4. DNA damage inhibition efficiency

DNA damage inhibition by aqueous extracts of *A. lanata* stem was tested by photolysing H_2O_2 by UV radiation in the presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA[27]. A total of 1 μL aliquots of pBR322 (200 μg/mL) were taken in three microcentrifuge tubes. A quantity of 50 μg of aqueous extracts was added to the tube. The remaining tube was left untreated as the irradiated controls. An amount of 4 μL of 3% H_2O_2 was added to all the tubes, which were then placed directly on the surface of a UV transilluminator (300 nm) for 10 min at room temperature. After irradiation, 4 μL of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added. The samples in all the tubes were analyzed by gel electrophoresis on a 1% agarose gel (containing ethidium bromide) in TBE buffer (pH 8). Untreated non-irradiated pBR322 plasmid (C) was run along with untreated UV-irradiated plasmid DNA (R) and aqueous extract-treated UV-irradiated sample (E).

2.6. Analysis of phenolic content

2.6.1. Estimation of total phenolic content

Total phenolic content of the aqueous extract of the stem of *A. lanata* was determined using the Folin-Ciocalteau reagent method[28]. The crude aqueous extract was diluted in methanol to obtain different concentrations (125, 250, 500 and 1000 μg). Extract (50 μL) was mixed with 2.5 mL of Folin-Ciocalteau reagent (1/10 dilution in purified water) and 2 mL of 7.5% Na_2CO_3 (w/v in purified water). The mixture was incubated at 45 ℃ for 15 min. The absorbance was measured at 765 nm. Na_2CO_3 solution (2 mL of 7.5% Na_2CO_3 in 2.55 mL of distilled water) was used as blank. The results were expressed as gallic acid equivalence (GAE) in μg. Each experiment was performed in triplicates at each concentration.

2.6.2. Determination of phenolic compounds: HPLC analysis

Analysis of phenolic compounds present within the aqueous extract of stem of *A. lanata* was performed using the phenolics reference standard for HPLC[29]. HPLC analysis was carried out using a Waters 2487 HPLC system consisting of a dual λ detector and a Waters 1525 binary pump, and equipped with a Waters Symmetry C18 column (5 μm, 4.6 mm × 150 mm) with Waters SentryTM universal guard column (5 μm, 4.6 mm × 20 mm) (Waters Corporation, Milford, MA, USA). Gradient elution was performed with solution A (50 mM sodium phosphate in 10% methanol; pH 3.3) and solution B (70% methanol) in the following gradient elution program: 0-15 min 100% of solution A; 15-45 min 70% of solution A; 45-65 min 65% of solution A; 65-70 min 60% of solution A; 70-95 min 50% of solution A; 95-100 min 0% of solution A. The dried extract was dissolved in HPLC grade methanol (10 mg/mL), filtered through sterile 0.22 μm syringe filter and 10 μL volume was injected to the HPLC. Flow rate was maintained 1 mL/min and temperature was adjusted to 25 ℃. Detection was monitored at diverse wavelengths 250, 280, 320, 370 and 510 nm.

2.7. Hemolytic activity

Hemolytic activity of aqueous extracts of *A. lanata* stem was performed by the spectroscopic method[30,31]. Five milliliters of blood were collected from healthy individual (blood group O positive) and erythrocytes were collected after repeated washing in sterilized phosphate buffer saline solution (pH 7.2±0.2). The cells were resuspended in phosphate buffer saline to 0.5%. A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL of the plant extracts (125, 250, 500 and 1000 μg/mL concentrations in normal saline). The mixtures were incubated for 30 min at 37 ℃. The mixture was centrifuged at 1500 rpm for 10 min in a cooling centrifuge. The free hemoglobin in the supernatants was measured in UV-Visible spectrophotometer at 540 nm. Phosphate buffer saline and distilled water was used as minimal and maximal hemolytic controls. Each experiment was performed in triplicates for each concentration.

The percentage hemolysis was calculated according to the

following formula:
Percentage hemolysis=[(At-An)/(Ac-An)] ×100
Here, At is the absorbance of test sample, An is absorbance of the minimal control, Ac is the absorbance of the maximal control

2.8. Statistical analysis

The values of DPPH radical scavenging activity, metal chelating activity, reducing power activity and hemolytic activity of the aqueous extract of *A. lanata* stems are expressed as mean±standard deviation of the response of three replicates per sample. Results were analyzed using Microsoft Excel 2007 (Roselle, IL, USA).

3. Results

3.1. Phytochemical screening

Primary phytochemical screening of the aqueous extract of the *A. lanata* stem revealed the presence of phenolic compounds, saponins, flavonoids, tannins and phytosterols, whereas, carbohydrates, proteins, oil and fats and alkaloids were not present in the extract. These phytochemical compounds are the key candidates in the medicinal value of this plant.

3.2. Antioxidant activity

3.2.1. DPPH radical scavenging activity

DPPH is a highly stable free radical with purple color. After reacting with an antioxidant it turned to a stable yellow color compound (diphenyl-picrythydrazine). Reduction in the color was measured by spectrophotometer (λ_{max} 517). In this study, aqueous extract of *A. lanata* stem exhibited high DPPH radical scavenging activity with an IC_{50} value 110.74 μg/mL and the free radical scavenging activity was found to be increasing with increase in the dose. The results are expressed as percentage inhibition of DPPH (n=3) and reported in Figure 1.

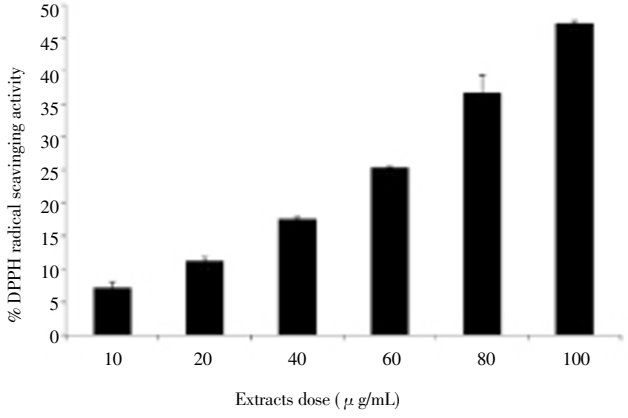

Figure 1. DPPH radical scavenging activity of varying concentrations of *A. lanata* stem aqueous extracts.

3.2.2. Metal chelating activity

Ferrozine quantitatively forms complexes with Fe^{2+} and produce red color. In the presence of chelating agents, the formation of this complex is disrupted, thereby impeding the formation of red color. Extract exhibited dose dependant metal chelating activity with an IC_{50} value 758.17 μg/mL. The results are expressed as percentage metal chelating activity (n=3). However, the metal chelating activity of ascorbic acid was higher than that of extract (Figure 2).

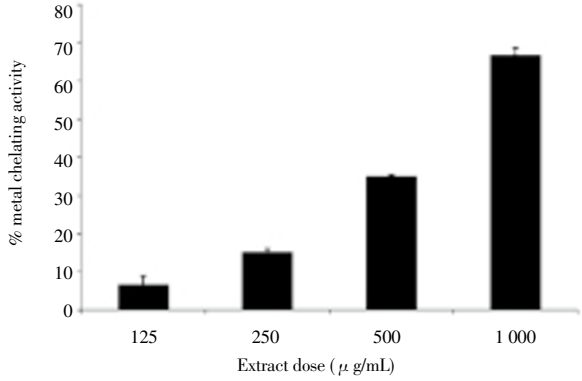

Figure 2. Metal chelating activity of varying concentrations of *A. lanata* stem aqueous extracts.

3.2.3. Reducing power activity

Reducing power activity is a good indicator of antioxidant activity. The plant having high reducing power generally reported to carry high antioxidant potential too. Reduction of Fe (Ⅲ) by electron-donating activity of the compounds reflects the possible antioxidant mechanism of the compound. In this experiment, Ferric ions are reduced to ferrous ions with the color of the reaction mixture changes from yellow to bluish green. The results for ferric reducing power activity of *A. lanata* stem extract with compared to ascorbic acid are reported in Figure 3. Extract exhibited dose dependent reducing power potential. However, the efficacy was found to be lower than that of ascorbic acid.

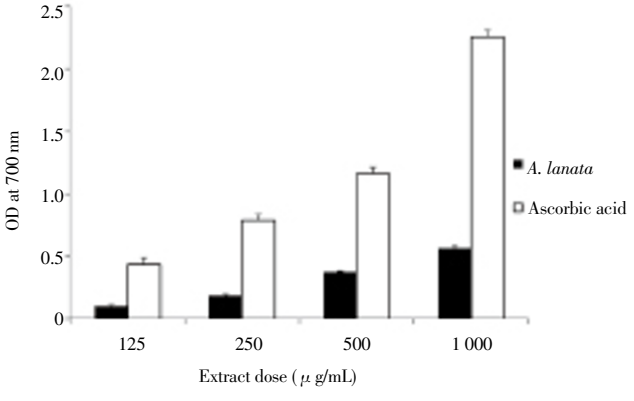

Figure 3. Reducing power activity of varying concentrations of *A. lanata* stem aqueous extract with comparision to ascorbic acid.

3.2.4. DNA damage inhibition efficiency

DNA damage inhibition efficiency of the extract is reported in Figure 4, it shows the electrophoretic pattern of pBR322 DNA following UV-photolysis of H_2O_2 in absence (in controls C and R) and presence (samples S) of the extract. Control pBR322 (C) showed a bands on agarose gel electrophoresis. UV-photolysis of H_2O_2 in R damaged the entire DNA (no bands visible). The extract displayed considerable protective activity and indicate the DNA band. The results infer that UV-photolysed H_2O_2 (3%) treatment of pBR322 obliterated the entire DNA (in R), while 50 μg of aqueous extract protected against DNA damage (S).

Figure 4. Effect of varying concentrations of *A. lanata* stem aqueous extracts on the protection of plasmid DNA (pBR322) against oxidative damage caused by UV-photolysed H_2O_2.
C=untreated non-irradiated DNA (C), CR=untreated UV-irradiated DNA (R) and aqueous extract treated DNA (S).

3.3. Analysis of phenolic content

3.3.1. Total phenolic content

In the current study, total phenolic content of the aqueous extract of *A. lanata* stem was estimated by Folin-Ciocalteau reagent method. Quantification was done with respect to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE) in μg and reported in Figure 5. Phenolic content of the extract showed dose dependent increases.

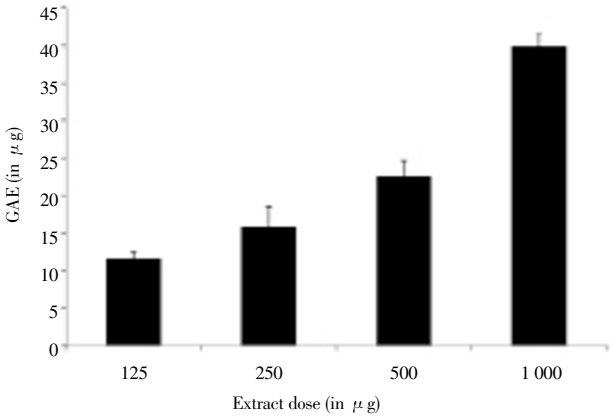

Figure 5. Total Phenolic content in varying concentrations of *A. lanata* stem aqueous extracts.

3.3.2. HPLC analysis for phenolic compounds

Phenolic compound is the large group of phytochemicals present in plant, so far more than 8 000 structurally known phenolic compounds have been reported[32]. High diversity and structural complexity make them difficult to identify. In this study, qualitative analysis of the different phenolic extract was performed by HPLC analysis. Some of the phenolic compounds present in aqueous extract of *A. lanata* stem were identified by comparison with the reference retention times as reported earlier[29]. From HPLC spectra, 3,4,5-OH (gallic acid), apigenin-7-*O*-glucoside (apigetrin), quercetin-3-*O*-rutinoside (rutin) and 3,5,7,3,4,5-OH (myricetin) were identified as major components in the extract (Table 1).

3.4. Hemolytic activity

Hemolytic activity of the aqueous extract of *A. lanata* stem was screened against normal human erythrocytes. The extracts showed very low hemolytic effect with an IC_{50} value of 24.89 mg/mL; however the hemolytic percentage increased with increase in dose (Figure 6). The low hemolytic effect of the extract suggests the less/no toxicity of the plant on human erythrocytes.

Table 1
Phenolic compounds identified in aqueous extract of *A. lanata* stem by HPLC.

Compounds	λ [a] (nm)	Et_R [b](min)	Rt_R [c] (min)
Gallic acid (3,4,5-OH)	250	4.433	5.8
Apigenin-7-*O*-glucoside (apigetrin)	320	49.942	50.7
Quercetin-3-*O*-rutinoside (rutin)	370	40.389	40.6
Myricetin (3,5,7,3',4',5'-OH)	370	48.233	49.3

[a] Wavelenghth for determination, [b] Experimental retention times, [c] Reference retention times[29].

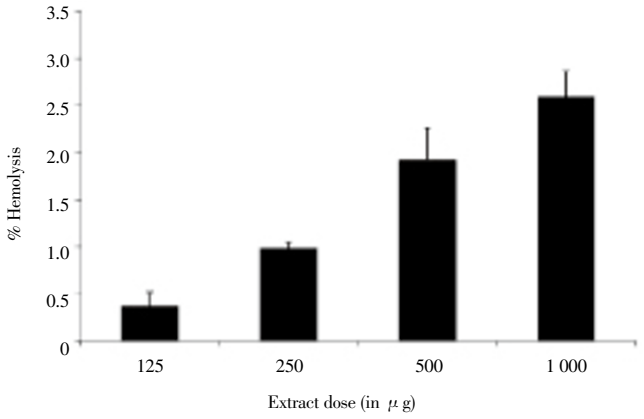

Figure 6. Hemolytic activities of varying concentrations of *A. lanata* stem aqueous extracts.

4. Discussion

Plants have been utilized as a natural source of medicines since thousands of years in Ayurvedic, Unani, Chinese and Nepalese, medication systems. Traditional Indian literatures such as Rig Veda, Yajur Veda, Atharva Veda, Charak Samhita and Sushrut Samhita reported the use of medicinal plants to antidote human diseases in ancient times. In last five decades, advancement of scientific technique and development of various analysis methods have changed the infrastructure of herbal drugs industries. Currently, scientists are focusing on the active compound from the natural sources to develop newer drugs. However, the preliminary pharmacological investigation is an important process to perform before or after the isolation of the bioactive molecule. Earlier, a variety of medicinal plants have been reported to possess different medicinal properties viz, anticancer activity[33], antimicrobial activity[34], antidiabetic activity[35], antioxidant activity[36], hepatoprotective activity[37], larvicidal activity[38], hemolytic activity[39], anti-inflammatory activity[40] *etc*. In this study, we have investigated the phytochemical composition, antioxidant activity and hemolytic activity of the aqueous extract of *A. lanata* stem by in vitro methods.

Phytochemicals are the non-nutritional plant compounds that are produced by the plants in self-defence to protect them from pest, microbes and environmental stress factors. Much of the medicinal property of any plant has been attributed by phytochemicals present within the plant. In past, herbs and plants have been reported to contain different phytochemical compounds with a wide range of activities, which may help in the development of new drugs[41-43]. Results of phytochemical study of aqueous extract of the *A. lanata* stem are in agreement with the previous studies where *A. lanata* extracts have been reported to possess glycosides, alkaloids and flavonoids as major phytochemicals[44-51]. Earlier, leaves of *A. lanata* reported to possess several nutritionally beneficial compounds such as carbohydrate, crude protein and ash, potassium, calcium, magnesium, zinc, ferrous and manganese. However in our study, the stem extract not exhibited the presence of proteins and carbohydrates[52].

In recent time, humans are living restless life in a polluted environment, eating junk foods, smoking cigarettes, drinking alcohol; all these factors enhance the free radical generation in body and resulted in oxidative stress[53]. Insufficient dietary antioxidant failed to control the oxidative stress and need to be supported by antioxidant supplements. Consumer rejection towards the synthetic antioxidants provides an opportunity to discover natural antioxidant. Thus in this study antioxidant potential of aqueous extract of *A. lanata* stem was analysed against a variety of free radicles by DPPH radical scavenging activity, metal chelating activity and reducing power activity. Extract exhibited high antioxidant activity against a variety of free radicals, it was observed that the change in extract concentration effect the degree of antioxidant activity during experiment, hence it can be said that the antioxidant activity is depending on the extract concentration. During DNA damage inhibition assay, free radicals generated by UV photolysing of H_2O_2 damage the pBR322 plasmid DNA. Pre treatment of DNA with extract showed complete protection of plasmid DNA by neutralising the free radicals. Commonly, free radicals mediate DNA damage can cause mutagenesis in the cells and initiate the development of cancer[1]. DNA protective potential of the extract can be capitalized to develop the drugs to control cancer. Polyphenolic compound are well accepted antioxidant compound reported from plants and vegetables[54]. The extract exhibited the presence of three major groups of polyphenolic compounds (phenolic compounds, flavonoids and tannins). Among them, phenolic compounds are the largest group of polyphenols accounted for the antioxidant activity of plants[55]. HPLC analysis of the crude extract showed the presence of four phenolic compounds including gallic acid, apigetrin, rutin and myricetin. These compounds are well known for antioxidant properties and possibly the active principle of the extract. Hemolytic activity could be used as a primary tool for studying the toxicity of the drugs as it provides primary information on the interaction between molecules and biological entities at cellular level. Extract treatment of human erythrocytes caused very less hemolysis that represent the non toxic effect of the extract towards human erythrocytes. Further studies to isolate active principles from these plants will be of great medicinal importance.

The results obtained in the study represented that the

aqueous extract of *A. lanata* stem contain a variety of phytochemical compounds, which can effectively protect the body from oxidative damage by free radicals scavenging activity and thus can be used as a potent source of natural antioxidant compounds. This report also indicates the nontoxic effect of the extract on human erythrocytes. In future, further studies could be conducted to establish the antioxidant mechanism of aqueous extract of *A. lanata* stem. With all these results, we can conclude that *A. lanata* stem can be used as a source of safe and natural antioxidant compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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