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Research Article

Study of Polyphenol Content and Anti- Oxidative Potential of *Tribulus terrestris* Dry Fruit Extract

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ABSTARCT

The dry fruit extract of *Tribulus terrestris* has been used in traditional medicine for various therapeutic applications. This study was intended to investigate its anti- oxidant potential using *in-vitro* assays. Initially, the total polyphenolic, flavonoid, and tannin content of the ethanolic extract of its dry fruit were tested since these compounds confer majority of the reducing power to plant extracts. The anti- oxidant potential of the extract was investigated using radical scavenging assays such as DPPH and ABTS, superoxide radical scavenging assay, and ferric reducing anti-oxidant power assay. The polyphenol content was found to be 6.65 $\pm$ 0.64 (Mean $\pm$ S.E.M.; n = 3) mg GAE/ g of extract, flavonoid content was 0.40 $\pm$ 0.02 (Mean $\pm$ S.E.M.; n = 3) mg RE/ g of extract, and tannin content was 4.09 $\pm$ 0.67 (Mean $\pm$ S.E.M.; n = 3) mg TAE/ g of extract. The results for anti- oxidant assay obtained were found to be comparable to ascorbic acid and butylated hydroxytoluene standards. The results indicate presence of polyphenol compounds which confer majority of anti- oxidant properties to plant extracts. Further *in-vitro* and *in-vivo* investigations would reveal the efficacy of the extract as a dietary source of anti- oxidants.

Keywords: *Tribulus terrestris*, polyphenols, radical scavenging.

INTRODUCTION

The cellular metabolic pathways are complex and well-regulated. Free radicals are continuously being produced in the human body as a result of the metabolic activities. Such free radicals are extremely damaging and interact immediately with biological molecules causing DNA strand modifications, lipid peroxidation, and protein structure modifications[1]. The body's innate defense mechanism against these free radicals includes glutathione redox reaction, activation of enzymes such as superoxide dismutase, catalase, reductase among others. In certain disorders, the defense mechanism fails to keep up with the rate of free radical production and such excess free radicals prove deleterious causing cardiovascular disorders, diabetes, and also cancer[1-3].

The food we consume provides anti- oxidants in a form which can be readily utilized by the cells to neutralize the excess free radicals before they cause much damage[4]. Fruits and vegetables contain polyphenolic compounds mostly as their secondary metabolities[4-7]. Various reports state that there is a direct correlation in the amount of polyphenolic compounds in plants and their measured anti-oxidant activities. As such, polyphenolic compounds contribute majorly to the exhibited anti- oxidant activity. Including more polyphenol- rich food in our diet is thus the most accessible way to provide anti- oxidants to the body to protect it from the ill- effects of free radicals[8, 9].

The traditional systems of medicine have relied on the use of a large number of plant species for their medicinal properties[10- 13]. Recent studies indicate that these plant sources are, at the same time, rich sources of anti-oxidants[14- 18]. One of such medicinal plants is *Tribulus terrestris* which is an annual plant with thorny fruits belonging to Zygophyllaceae family growing mostly in warm conditions. Traditionally, the plant is used as an aphrodisiac, as a tonic, analgesic, diuretic and for treatment of urinary tract infections[19]. In the present study, we attempted to investigate the polyphenolic content of the ethanolic extract of the dry fruit of *T. terrestris* and study its anti- oxidant potential using *in-vitro* techniques.

MATERIALS AND METHODS

The dry fruits of *Tribulus terrestris* were purchased from a local Ayurvedic medicine shop. The required reagents such as sodium carbonate, sodium hydroxide, ethanol, L-ascorbic acid (ascorbic acid), butylated hydroxytoluene (BHT), 2, 2- diphenyl-1- Picrylhydrazyl (DPPH), 2, 2'-Azino- bis (3- ethylbenzothiazoline-6- sulfonic acid) Diammonium salt (ABTS), potassium persulfate, potassium dihydrogen phosphate, di- potassium hydrogen phosphate, potassium fericyanide, tricholoacetic acid, ferric chloride, nitro blue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (β- NADPH), Tris(hydromethyl)amonimethane (Tris) were purchased from Sisco Research Laboratories Pvt. Ltd. Shimadzu spectrophotometer (UV- 1800) present in the Molecular and Genetic Laboratory was used for all spectrometric

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measurements. Phenazine methosulfate (PMS) was purchased from MP Biologics. Folin- Ciocalteau reagent, rutin, tannic acid, sodium nitrite and aluminium chloride were provided by the Department of Pharmacology; while gallic acid was gifted by Department of Biochemistry.

Extraction of plant materials

The dried fruits of *T. terrestris* were powdered using mortar pestle. 10 gram (g) of the powdered fruit was extracted in 200 millilitres (ml) of ethanol using a Soxhlet apparatus. After four extraction cycles, the powdered fruit appeared colorless and the extract was collected. The dried extract was stored in -20 degree Celsius (^{o} C) freezer. During experiments, the extract was re-suspended in ethanol and utilized.

Total phenolic content estimation

Gallic acid was used as the standard for determining the total phenolic content using the Folin- Ciocalteau method. The method was modified as follows[20]. 12.5 microlitres (µl) of standard solution in ethanol of varying concentration was added to 900 µl water with 60 µl Folin-Ciocalteau reagent and vortexed. After 5 minutes, 170 µl of 7.5 % sodium carbonate solution was added, mixed by vortexing and incubated at room temperature for 90 minutes. Absorbance was measured using a spectrophotometer at 750 nanometer (nm) wavelength. The blank tube contained 12.5 µl of ethanol. The standard curve was constructed using gallic acid concentrations from 40 microgram/ ml (µg/ ml) to 200 µg/ ml in ethanol. The extract solution was diluted in ethanol to obtain absorbance within the standard curve values. Each solution was prepared in triplicate in three separate experimental repeats. The total phenolic content was calculated as mg gallic acid equivalent per gram of extract (mg GAE/ g of extract).

Estimation of tannin content

For the estimation of amount of tannins in the extract, tannic acid was used as the standard for the Folin-Ciocalteau method[20]. The tannic acid dilutions of 20 µg/ ml to 100 µg/ ml were prepared in ethanol and 10 µl of each solution was added to 740 µl water. 50 µl Folin-Ciocalteau reagent, 200 µl of 17.5 % sodium carbonate solution was added, vortexed, and incubated at room temperature for 30 minutes after which absorbance was measured at 725 nm. The blank tube contained 10 µl ethanol instead of standard solution, followed by similar additions as the standard dilutions as mentioned. The standard curve was plotted with concentration of the standard against their absorbance. The extract solution was diluted in ethanol accordingly and the tannin content was estimated as mg of tannic acid equivalent/ g of extract (mg TAE/ g of extract). Each solution was prepared in triplicate for three separate experimental repeats.

Estimation of flavonoid content

The aluminum chloride assay was used for determining the quantity of flavonoids in the extract using rutin as the standard[20]. Briefly, rutin dilutions ranging from 20 µg/ ml to 100 µg/ ml were prepared in ethanol. 100 µl of each dilution was added to a microfuge tube containing 400 µl water and 30 µl of 5 % sodium nitrite. After 5 minutes, 30 µl of 10 % aluminium chloride was added and mixed by vortexing. 200 µl of 1 molar (M) sodium hydroxide and 240 µl water was added, mixed and absorbance was measured at 510 nm using spectrophotometer. The blank tube contained 100 µl ethanol instead of standard solution. The extract solution was diluted in ethanol appropriately and flavonoid content was estimated from the standard curve plotted using concentration and absorbance values of rutin. The flavonoid content was represented as mg of rutin equivalent/ g of extract (mg of RE/ g of extract). Each solution was prepared in triplicate for three independent experimental repeats.

DPPH radical scavenging assay

The DPPH free radical scavenging assay was performed to study the free radical scavenging activity of the extract[21]. As standards, ascorbic acid (range 5 µM to 30 µM) and BHT (range 10 to 500 µM) dilutions were prepared in ethanol in a final volume of 250 µl. The extract dilutions from 0.1 µg/ ml to 40 µg/ ml were prepared in ethanol in a final volume of 250 µl. To each tube 750 µl of 200 µM DPPH solution in ethanol was added, mixed by vortexing and incubated at room temperature in the dark for 30 minutes following which absorbance was measured at 517 nm. The control tube contained 250 µl of ethanol instead of the samples and treated the same way as the standards and samples. Each solution was prepared in triplicate for two independent experimental repeats. The percentage inhibition or scavenging (% scavenging) of the DPPH radical was calculated as follows:

% Inhibition = [(Absorbance of control – Absorbance of sample)/ (absorbance of control)]*100

ABTS radical scavenging assay

The ABTS radical scavenging assay was performed according to earlier published methods with some modifications[22]. Ascorbic acid (dilution range 2 – 32 µg/ ml) and BHT (dilution range 5 – 80 µg/ ml) solutions in ethanol were used as standards. Extract dilutions (dilution range 4- 40 mg/ ml) were prepared in ethanol in a final volume of 0.2 ml. For production of $ABTS^{\cdot +}$ radical, 7 mM ABTS solution with 2.4 mM potassium persulfate was incubated at room temperature in dark for 16 hours. The absorbance of the $ABTS^{+}$ solution at 734 nm was adjusted to 0.7 and 1 ml of this solution was added to 0.2 ml of standard and sample dilutions in ethanol. The solution was mixed by vortexing and incubated at room temperature for 30 minutes, following which absorbance was measured at 734 nm. The control tube contained 0.2 ml ethanol instead of sample or standard and was treated in a similar way. Each solution was prepared in triplicate for two independent experimental repeats. The percentage inhibition or scavenging (% scavenging) of the $ABTS^{+}$ radical was calculated as follows:

% Inhibition = [(Absorbance of control – Absorbance of sample)/ (absorbance of control)]*100

Superoxide radical scavenging assay

For the generation of superoxide free radicals, the NADPH- PMS method was used for the reduction of NBT[23]. Ascorbic acid (dilution range 20 – 100 µg/ ml) and BHT (dilution range 0.1 –0.5 mg/ ml) solutions in ethanol were used as standards. Extract dilutions (dilution range

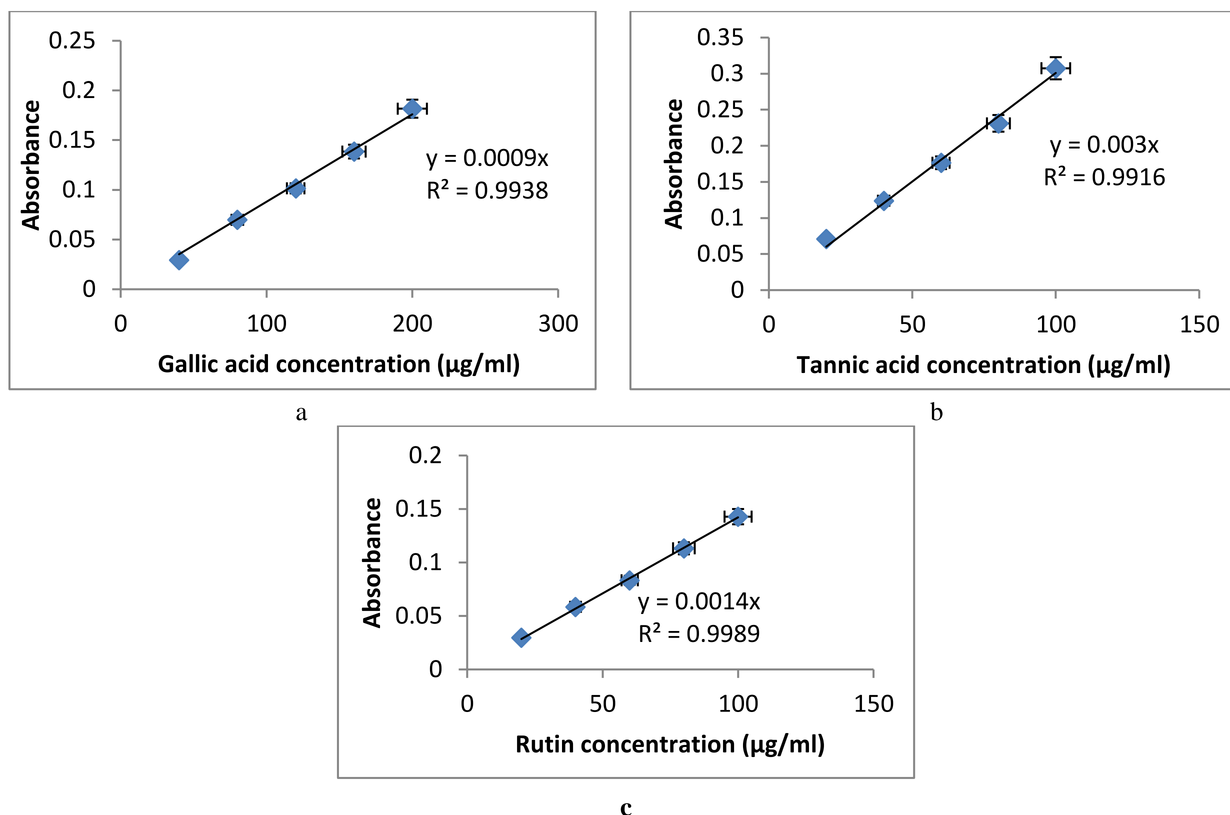

Figure 1: Calibration curve for determination of total phenolic content, tannin content, and flavonoid content. (a) Graph of gallic acid concentration (µg/ ml) against the measured absorbance at 550 nm. (b) Graph of tannic acid concentration (µg/ ml) against the measured absorbance at 725 nm. (c) Graph of rutin concentration (µg/ ml) against the measured absorbance at 510 nm.

0.5 - 5 mg/ ml) were prepared in ethanol in a final volume of 0.1 ml. Briefly, 0.1 ml of 1.13 mM NADPH, 0.25 ml of 500 µM NBT, 0.35 ml Tris-HCl (pH 8.0) were added to a tube containing 0.1 ml of standard or sample dilutions. The solution was mixed by vortexing and the reaction was initiated by adding 0.2 ml of 40.3 µM PMS. The tube contents were mixed and incubated at room temperature for 5 minutes following which absorbance was measured at 560 nm. The control sample contained 0.1 ml ethanol instead of sample or standard. Each solution was prepared in triplicate for two independent experimental repeats. The superoxide radical scavenging activity was expressed as percentage inhibition as follows:

% Inhibition = [(Absorbance of control – Absorbance of sample)/ (absorbance of control)]*100

Ferric reducing antioxidant power

To investigate the total reducing power of the extract, the ferric reducing antioxidant power assay was performed[24]. Ascorbic acid (dilution range 2 – 32 µg/ ml) and BHT (dilution range 5 – 80 µg/ ml) solutions in ethanol were used as standards. Extract dilutions (dilution range 1 - 20 mg/ ml) were prepared in ethanol in a final volume of 0.1 ml. 0.25 ml of phosphate buffer (pH 6.6), 0.25 ml of 1 % potassium ferricyanide were added to a tube containing 0.1 ml of standard or sample in ethanol. The mixture was incubated at 50 °C for 20 minutes and after cooling 0.25 ml of 10 % trichloroacetic acid was added. The samples were then centrifuged at 5000 rpm for 10 minutes and the upper layer was added to a separate tube containing 0.25 ml water and 0.05 ml 0.1 % ferric chloride. The mixture was incubated at room temperature for 10 minutes following which absorbance was measured at 734 nm. The control tube contained 0.1 ml ethanol instead of standard or sample. The reducing power was estimated by plotting the concentration of samples against the measured absorbance at 734 nm. Each solution was prepared in triplicate for two independent experimental repeats.

RESULTS AND DISCUSSION

The polyphenolic compounds found in plants are mostly secondary metabolites which confer certain characteristics to the plant. When such polyphenolics containing food is consumed, these compounds have been proven to be beneficial in many ways including protection against oxidative stress by scavenging the harmful free radicals produced in the body or by inducing expression of factors in the cell which help in abating the oxidative damage caused by such free radicals[5]. The content of polyphenolics in plant extract samples has been positively co-related to its observed anti-oxidant activity measured *in- vitro*[7, 14-18]. As such, studying the content of polyphenolic compounds in a given plant extract sample can prove to be informative while studying its anti-oxidant activities. The term polyphenol compound encompasses large groups of compounds such as flavanoids, tannins, anthocyannins, catechins, lignans, etc. having diverse structures[5]. Flavanols and flavones found in pomegranate have anti-cancer properties[25-27]. Tannins and flavonoids have anti-

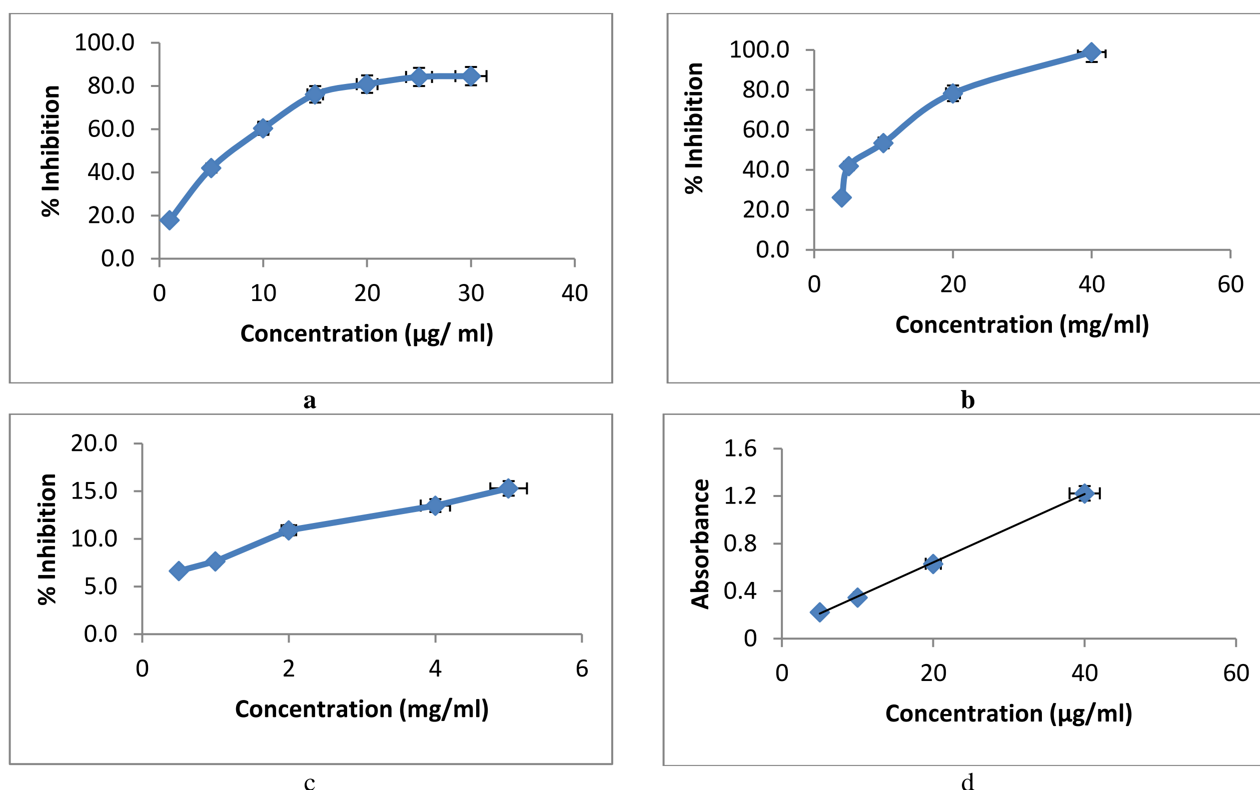

Figure 2: Graph of anti- oxidant activity of extract against the respective concentration studied using different anti-oxidant assays such as (a) DPPH radical scavenging assay; (b) ABTS radical scavenging assay; (c) Superoxide radical scavenging assay; (d) Ferric reducing anti- oxidant power assay.

oxidant, anti- inflammatory, anti- fungal and healing properties[28]. Catechins found in tea have cytoprotective activity against oxidative stress induced toxicity and are known to have anti-angiogenic and anti- tumour properties[29].

The total phenolic content analysis was performed using the Folin – Ciocalteu method. The Folin- Ciocalteau reagent is made up of sodium molybdate and sodium tungstate which form complex with polyphenols in alkaline conditions. Higher the amount of reacting polyphenolic groups under given conditions higher is the amount of complex formed and higher the absorbance. For measuring the total phenolic content, gallic acid was used as the standard to plot the calibration curve and the phenolic content of the extract was plotted accordingly. The obtained total phenolic content was expressed as gallic acid equivalent per gram dry weight of the extract. The total phenolic content of the extract was determined to be 6.65 $\pm$ 0.64 (Mean $\pm$ S.E.M.; n = 3) mg GAE/ g of extract [Fig. 1-a]. The tannin content of the extract was also determined using the Folin- Ciocalteu method under slightly modified conditions and tannic acid was used to plot the standard curve. The calculated tannin content of the extract was expressed as tannic acid equivalent per gram dry weight of the extract. The tannin content was found to be 4.09 $\pm$ 0.67 (Mean $\pm$ S.E.M.; n = 3) mg TAE/ g of extract [Fig. 1- b].

The flavonoid content of the extract was determined using the aluminum chloride method. Varying concentrations of rutin were used to plot the calibration curve and the flavonoid content of extract was calculated as mg rutin equivalent per g dry weight of the extract. The flavonoid content was found to be 0.40 $\pm$ 0.02 (Mean $\pm$ S.E.M.; n = 3) mg RE/ g of extract [Fig. 1-c].

After studying the polyphenolic contents, the anti-oxidant potential of the extract was studied using various free radical scavenging assays. One of the methods employed the use of stable free radical, DPPH, with maximum absorbance at 517 nm. The polyphenolic compounds in the extract neutralize the stable free radical DPPH resulting in discoloration of the solution proportional to the amount of radicals neutralized[21]. Ascorbic acid and BHT were used as the standards to compare the anti-oxidant potential of the extract. Ascorbic acid exhibited 90 % inhibition at 30 µM; while BHT inhibited 88 % of the DPPH radical at 350 µM concentration. The extract solution showed increasing inhibition corresponding to increase in its concentration with maximum inhibition of 84.6 % at 30 µg/ ml concentration [Fig. 2- a].

The ABTS radical scavenging assay relies on the production of $ABTS^{\cdot +}$ cation by reacting ABTS with potassium persulfate resulting in a blue/ green solution. The subsequent reduction of the radical $ABTS^{\cdot +}$ cation by anti- oxidants results in discoloration of the solution which can be measured as decrease in absorbance at 734 nm[22]. Ascorbic acid and BHT solutions exhibited complete inhibition at 32 µg/ ml and 80 µg/ ml, respectively. The extract solution was observed to have a proportional increase in inhibition with increasing concentration, although at higher concentration compared to standards,

with complete inhibition at 40 mg/ ml concentration [Fig. 2-b].

In the superoxide radical scavenging assay, superoxide radical produced by using the NADPH/ PMS system reduces the tetrazolium salt NBT to insoluble blue colored formazan crystals. The anti-oxidants prevent the reduction of NBT by scavenging the superoxide radicals and thus resulting in decrease in absorbance measured at 560 nm[23]. Ascorbic acid and BHT used as standards showed 30.9 % inhibition at 100 µg/ ml and 12.5 % at 500 µg/ ml, respectively. The extract solution showed increasing scavenging activity with corresponding increase in concentration from 6.6 % inhibition up to a maximum of 15.3 % inhibition at 5 mg/ ml [Fig. 2- c]. It was observed that above 5 mg/ ml concentrations of the extract, the solution turned turbid which interfered with the absorbance measurements.

The ferric reducing antioxidant assay is an indirect measure of the samples' anti-oxidant potential indicated by reduction of ferrous (Fe^{3+}) ions in the solution to ferric (Fe^{2+}) ions which can be measured by a change in absorbance[24]. Ascorbic acid and BHT standard solutions exhibited a proportional linear increase in absorbance with increasing concentration across the entire concentration range. Similarly, the sample also showed increased absorbance with corresponding increase in concentration from 5 mg/ml to 40 mg/ ml. Extract solutions with concentration greater than 40 mg/ ml were turbid and hence could not be tested [Fig. 2- d].

CONCLUSION

Based on the results obtained from the above described experiments, it can be seen that the ethanolic extract of the dry fruit of *T. terrestris* contains measurable polyphenolics comprising of flavonoids, and tannins among others. As mentioned, there is a positive relation between the proportion of polyphenolic content of plant extracts and its anti- oxidant potential[7, 14- 18]. This anti- oxidant potential was successfully studied using the above methods which indicate that the extract has anti- oxidant potential comparable to the standards used. Further *in-vitro* and *in-vivo* experiments with a detailed phytochemical screening might shed light on the use of this extract as a source of anti- oxidants for human use.

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