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Original article

In vitro antioxidant and cytotoxic potential of methanolic extracts of selected indigenous medicinal plants

Abida Kauser[1], Syed Muhammad Ali Shah[2], Naeem Iqbal[3], Mian Anjum Murtaza[4], Iqbal Hussain[3], Asma Irshad[5], Suhaila Nasir[1], Muhammad Akram[2], Naveed Munir[6], Muhammad Riaz[7]

[1]Department of Botany, Government College Women University Faisalabad, Pakistan; [2]Department of Eastern Medicine and Surgery, Government College University Faisalabad, Pakistan; [3]Department of Botany, Government College University Faisalabad, Pakistan; [4]Institute of Food Science and Nutrition, University of Sargodha, Sargodha-Pakistan; [5]Centre of Excellence in Molecular Biology, University of the Punjab, Lahore-Pakistan; [6]Department of Biochemistry, Government College University Faisalabad, Pakistan; [7]Department of Allied Health Sciences, Sargodha Medical College, University of Sargodha, Sargodha-Pakistan - E-mail: riazmlt786@gmail.com

Summary. Plants of medicinal importance are the source of various bioactive compounds. These compounds include the antioxidants which play significant role in preventing oxidative stress caused by the reactive oxygen species (ROS). The present research work was conducted to evaluate the methanolic extracts of selected indigenous medicinal plants for their antioxidant activities by different assays as well as their cytotoxic activity. Methanolic extracts of five selected medicinal plants viz *T. chebula; E. officinalis, T. natans, M. oleifera,* and *T. belenica* were prepared and tested for their antioxidant and cytotoxic activities. Total Flavonoid content (TFC) was found to be higher in methanolic extract of *E. officinalis* (43.12±2.45 µg CE/g) followed by *T. natans* (21.32±1.06 µg CE/g), *T. chebula* (15.22±0.63 µg CE/g), *T. belenica* (13.36±0.78 µg CE/g) and *M. oleifera* (5.21± 0.75 µg CE/g). Among the studied medicinal plants, highest total phenolic contents (TPC) were found in methanolic extract of *E. officinalis* (475.5±14.3 µg GAE/g) while the lowest content was found in *M. oleifera* extract (170.9±5.6 µg GAE/g). The studied plants also showed good radical scavenging activity through DPPH assay and reducing potential through FRAP assay. On hemolytic assay, minimal percent hemolysis of washed RBCs was shown by methanolic extract of *E. officinalis* (5.89±0.78 %) while highest percent hemolysis was observed in washed red cells treated with *T. chebula* (13.12±1.2 %). Triton X-100 and normal saline were used as positive and negative controls showing 87.3±8.64 % and 0 % hemolysis, respectively. The current study results indicate that the studied medicinal plants posses considerable antioxidant substances responsible for antioxidative and cytoprotective potential.

Key words: antioxidant activity, *Moringa oleifera*, *Terminalia chebula*, *Emblica officinalis*, *Trapa natans* and *Terminalia belerica*

Introduction

Pakistan is blessed with the wealth of medicinal plants and is considered one among few spots in the globe richly supplied with fascinating bio-assortments. About 80% of global population relies on the conservative ways of serving the humanity. Almost 6000 plant species are found in Pakistan having healing potential against various ailments (1). Pakistan is considered among those nations where the system of Unani medication is practiced among the majority of its populace. The system of Unani medication was started in Greek by the primitive old fashioned rationalists and then adopted by the Muslims during amid superb time of

Islamic human improvement. The Muslim scholars brought it to the subcontinent and famously by Muslim practiced for the ages until modern medication system. Medicinal plants are the main donor of antioxidative substances prohibiting the ROS induced oxidative stress. Free radical scavenging activity plays its role in assisting the organisms to cope with oxidative stress destroying the free radical induction process. It has been widely accepted that ROS induces oxidative stress causing destruction to cells leading the various human ailments such as disorders of the central nervous system, metastatic carcinoma, arthritic problems as well as aging. The plant antioxidant compounds are effective against the harmful effect of ROS (2, 3). A variety of ingredients/substances present in plant tissues which are vital certain physiological events of human body. Due to the presence of active metabolites, therapeutic plants have incredible importance for the management of contagious diseases (3). Plants rich with polyphenolic metabolites have certain biological activities like antiviral, antithrombotic, anti-carcinogenic, anti-allergic, antimicrobial, hepatoprotective anti-hypertensive activities (4). Plant kingdom contains several vibrant parts that are being used in curing of various extreme afflictions (5). Ancient study indicated that the plants were gathered for the use of physical solutions by antiquated individuals. Numerous substances called optional metabolites present in therapeutic plants were utilized for medication against various illnesses (3). Plants based drugs are being utilized regularly by the cultivator for the treatment of various ailments (6). Antioxidants are substances that can scavenge the free radicals inhibiting the oxidative processes leading to the cure from oxidative pathology (7, 8). Gershaman and Gilbert in 1968 suggested that the free radicals produced are because of the high demand of oxygen for all life forms brought a vast majority of unsafe impacts. This thought did not raised the attention of most of the scientists until 1968 with the discovery of a chemical causing the reactant expulsion of an oxygen radical (9). Pakistan is richly supplied with restorative herbs having a fluctuated atmosphere but scattered over an widespread area (10). In modern system of medication, medicinal plants playing an important role in providing model atoms to plausible development into routine medications by the pharmaceutical stakeholders.

Therefore the current research was conducted to evaluate the antioxidant potential of methanolic extracts of selected indigenous medicinal plants and their cytotoxic effect on washed red cells through hemolytic assay.

Material and methods

Experimental work of present research has been done in Bioassay Section, Medicinal Biochemistry Research Lab in Department of Biochemistry, University of Agriculture, Faisalabad. Medicinal plants for the present study were purchased from the local market of Faisalabad, Pakistan. Plants and their different parts were taxonomically identified and authenticated from Department of Botany, University of Agriculture, Faisalabad. In the present study *Moringa oleifera (Suhnjina)* seed, *Terminalia chebula (Herer)* fruit, *Emblica officinalis (Amla)* fruit, *Trapa natans (Singhara)* fruit, *Terminalia belerica (Halila)* fruit were used.

The selected plants materials were cleaned using distilled water, shade dried and ground into fine powder using mortar and pestle. The powdered plants materials (1 Kg) was extracted in aqueous methanol (70:30 v/v) using an orbital shaker (Gallenkamp, UK) for 72 hours at 25-28°C. Using Whatman No. 1 filter paper the extract was filtered and the filtrate was evaporated at 45-50°C under reduced pressure, in a rotary evaporator (Heidolph, model Laborata 4000, Schwabach, Germany). The concentrated extracts were weighed and percentage yield of extract (g/100 g of dry plant) was calculated.

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \text{ X } 100$$

Antioxidant potential of medicinal plants extracts

Antioxidant potential of the selected plants extract was determined by using different antioxidant assays.

Total phenolics and flavonoids contents

The total phenolics contents (TPC) were determined using Folin-Ciocalteu (FC) reagent as described

by Jain *et al*. (11). Plants extract (1mL; 0.001g/mL) was mixed with FC reagent and optical density of blue colored complex was taken at 765nm after 1 hour using 96 well Microplate reader, Model µ-QuantTM, from BioTek, USA. All determinations were performed in triplicates. Gallic acid was used as the standard (Figure 1). Total phenolic contents of studied plants extract was quantified as gallic acid equivalents (GAE) and calculated using the formula.

$$\mathbf{T = C \times V / M}$$

Where,

T = Total phenolic compounds present in 1 g of plants extract as mg GAE.

C = Gallic acid concentration (mg/mL) obtained from standard curve.

V = Extract volume (mL).

M = Extract weight (grams).

The total flavonoids contents (TFC) were assayed following Pranuthi *et al*. (2014)(12). Briefly plants extract (0.5mL) was added to 2mL distilled water and 0.15mL of 5% $NaNO_2$ solution and incubated for 6 minutes. After adding 0.15mL of 10% $AlCl_3$ the mixture was incubated for 6 min followed by the addition of 4% NaOH solution and methanol to make the final volume upto 5mL. Optical density of the reaction mixture was taken at 510nm after 15minutes incubation. Catechin was used as standard (Figure 2). Total flavonoid contents (TFC) of extracts were quantified as µg catechin equivalents per gram of extract.

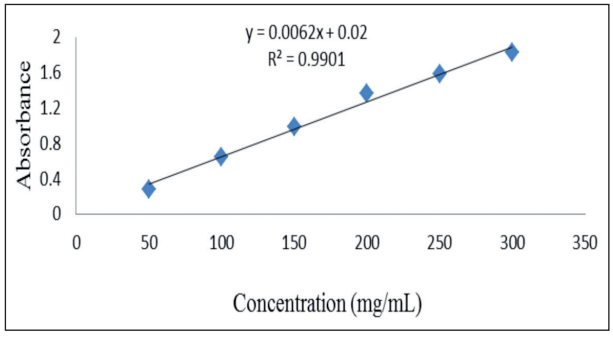

Figure 1. Standard curve of Gallic acid for TPC.

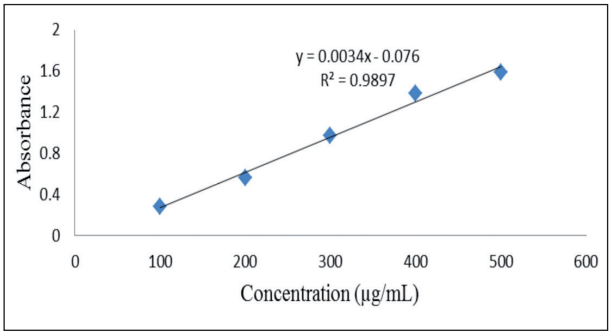

Figure 2. Standard curve of Catechin for TFC.

DPPH Scavenging Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was used for the determination of radical scavenging activity as antioxidant potential of plant extracts (8). For this 1mL of 0.004% DPPH in methanol solution was mixed with plants extract (3mL) and then mixture solution was kept under dark environment for 30 minutes. The optical density at 517 nm was measured which is inversely related to radical scavenging activity. Vitamin C (ascorbate) was used as standard. The reagent solution without plants extract was used as procedural control. The percentage inhibition was calculated.

$$\text{DPPH Inhibition (\%)} = \frac{\text{Blank absorbance } (A_0) - \text{Sample absorbance } (A_1) * 100}{\text{Blank absorbance } (A_0)}$$

Where

A_1 = Absorbance of sample.

A_0 = Absorbance of blank.

Measurement of reducing power

The ferric reducing antioxidant potential (FRAP) assay based on the principle of direct electron donation reducing the Fe^{3+} $(CN)_6$ to Fe^{2+} $(CN)_6$ form was used for the measurement of reducing potential of plants extract. The reaction mixture contained 1mL of plants extract, phosphate buffer (2.5mL 0.2M; pH 6.6) and 1% potassium ferricyanide (2.5mL). After 20 minutes incubation at 50°C, stopped the reaction by adding 2.5mL TCA (1% w/v) following centrifugation at 3000 rpm for 10 min. Supernatant (2.5mL) was added

to 2.5 mL distilled water and 0.5 mL ferric chloride (0.1% w/v) and the optical density was taken at 700 nm.

Cytotoxic activity by Hemolytic assay

The cytotoxic potential of plants extract was determined through hemolytic assay following the protocol of Powell *et al*. (13). Human blood (3mL) was taken in a sterile falcon tube of 15 mL capacity and washed with 5ml chilled PBS three times by centrifuging the tubes for 5mins. In 2 mL eppendorf tubes, 180 µL of RBCs suspension was mixed with 20 µl of plants extract. Tubes were centrifuged for 5 min and the supernatant (100 µL) was diluted with 900 µL chilled PBS. Triton X-100 (0.1%) was used as positive control for complete hemolysis of erythrocytes and PBS as negative control. Measured the absorbance at 576 nm using 96 well Microplate reader, Model µ-QuantTM, from BioTek, USA.

Data analysis

The data obtained were computed using Excel spread sheet, Microsoft word version 2007. Mean and SD (standard deviation) values of each tested parameters were calculated using Excel spread sheet, Microsoft office version 2007 for Windows.

Results and discussion

The present research work was conducted to evaluate the methanolic extracts of selected indigenous medicinal plants for their antioxidant activity by different antioxidant assays as well as their cytotoxic activity through hemolytic assay. Methanolic plant extracts of *T. chebula; E. officinalis, T. natans, M. oleifera,* and *T. belenica* were used in this study. Among the studied plants methanolic extracts, highest percent yield (g/100 g dry weight) was obtained from *T. chebula* fruits (26.32%) followed by *T. belenica* fruits (18.8%), *E. officinalis* fruits (13.74%), *M. oleifera* seeds (9.4%) while *T. natans* fruits gave the least yield (5.14%) as shown in Figure 3.

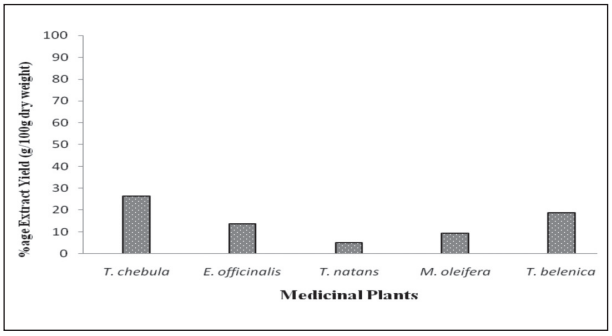

Figure 3. Percentage yield of selected medicinal plants methanolic extract.

Antioxidant potential of methanolic plants extract

Antioxidants are the chemicals or agents that have the ability to scavenge the ROS or free radicals. The medicinal plants are the source of natural antioxidative substances which helps improve the antioxidant capacity of living body. Due to the importance of natural antioxidants, we in the present study determined the antioxidant potential of studied plants extracts prepared in methanolic solvent.

Table 1. Antioxidant potential of methanolic extract of selected medicinal plants.

Plants/parameters	TFC (µg CE/g) Mean±SD	TPC (µg GAE/g) Mean±SD	Scavenging activity (%) Mean ±SD
T. chebula	15.22±0.63	359.8±8.47	89.9±6.4
E. officinalis	43.12±2.45	475.5±14.3	90.11±7.2
T. natans	21.32±1.06	324.77±10.4	66.21±4.6
M. oleifera	5.21±0.75	170.9±5.6	83.42±6.3
T. belenica	13.36±0.78	440.07±15.9	63.23±5.8

Values were expressed as Mean± SD

Note: TFC (Total Flavonoid Contents), TPC (Total Phenolic Contents), % (Percent), SD (Standard Deviation)

Total phenolic and flavonoids contents

The most commonly reported phytochemicals in medicinal plants extract have been found possessing many biological activities, the most important of which is the antioxidant potential. The total flavonoid and phenolic contents of selected medicinal plants were investigated in the present study and results as Mean±SD (Standard Deviation) are presented in Table 1. All the selected medicinal plants have variation in the contents of these phytochemicals in methanolic solvent extract. Among the tested medicinal plants, highest flavonoid contents was found in methanolic extract of E. *officinalis* (43.12±2.45 µg CE/ g dry weight) followed by methanolic extract of *T. natans* (21.32±1.06 µg CE/g), *T. chebula* (15.22±0.63 µg CE/g), *T. belenica* (13.36±0.78 µg CE/g) and *M. oleifera* (5.21±0.75 µg CE/g). Catechin was used as the standard and hence the results were expressed as Catechin Equivalents (CE). The total phenolic content was also determined in the methanolic extract of selected medicinal plants and the results were taken as Gallic Acid Equivalents (GAE). The maximum phenolic content was found in methanolic extract of E. *officinalis* (475.5±3.6 µg GAE/g dry weight) while the least phenolic content was found in *M. oleifera* (170.9±2.59 µg GAE/g dry weight). Moderate phenolic contents were found in *T. belenica, T. chebula* and *T. natans* in the order of 440.07±3.0, 359.8±3.47 and 324.77±6.52 µg GAE/g dry weight of plant material, respectively. These phytoconstituents have antioxidant potential not because of the electrons donating ability of hydrogen only but also because of their stable radical intermediates. The literature survey revealed the correlation between antioxidant role of phenolic compounds to antioxidant potential of medicinal plants (14, 15). So the plants having good antioxidant potential might be the rich source of phenolic compounds.

Radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used for the evaluation of free radical scavenging activity of the methanolic extracts of selected medicinal plants in current research study. The DPPH assay is the most commonly used method for determining the radical scavenging ability of medicinal plants extracts and other extracts from natural source as a marker for evaluation of antioxidant potential of tested material. Methanolic extract of E. *officinalis* exhibited the highest radical scavenging potential with percent inhibition of 90.11±7.2 than the other test methanolic extract of selected medicinal plants in this study. While the lowest percent inhibition of free radical production was shown by methanolic extract of *T. belenica* with an average±SD value of 63.23±5.8. The other selected plants methanolic extract showed percent inhibition of free radicals in the order of *T. chebula*, *M. oleifera* and *T. natans* with an average±SD values of 89.9±6.4, 83.42±6.3 and 66.21±4.6, respectively. The results of the scavenging activity of selected plants methanolic exract was in favor of the implication of flavonoids and phenolic compounds as the potential source of antioxidants in the selected medicinal plants responsible for the antioxidant activity by hydrogen transferring reactions.

The results of the present study indicates that higher the flavonoids and phenolics in the methanolic plants extract, higher is the percentage inhibition/free radical scavenging activity of the plants extract therefore greater is the antioxidant potential of the plants extract. Here among the tested plants extract E. *officinalis* exhibited higher flavonoid and phenolics content and also the highest percent inhibition of free radicals production, hence is the richest in antioxidants among the tested medicinal plants.

Reducing power

Different methods are being practiced to assay antioxidant capacity of extracts from natural sources. But the rapid ferric reducing antioxidant power (FRAP) assay has been gaining more attention as a tool for determining the antioxidant potential of medicinal plants extract. In the present study, we also used the FRAP method for the reducing power determination of selected medicinal plants methanolic extracts. In FRAP assay, the reduced ferrous Fe^{2+} formed from Fe^{3+}ferricyanide complex by the action of antioxidants and this can be evaluated by determining the navy blue color formation at 700 nm (16).

In current study, the strongest reducing power exhibited by methanolic extract of *T. natans* while the weak reducing power was observed in methanolic extract of *E. officinalis* among the tested medicinal plants methanolic extract (Fig. 4).

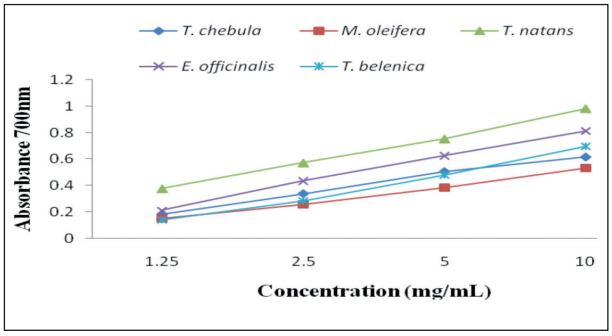

Figure 4. Reducing power of methanolic extracts of selected medicinal plants.

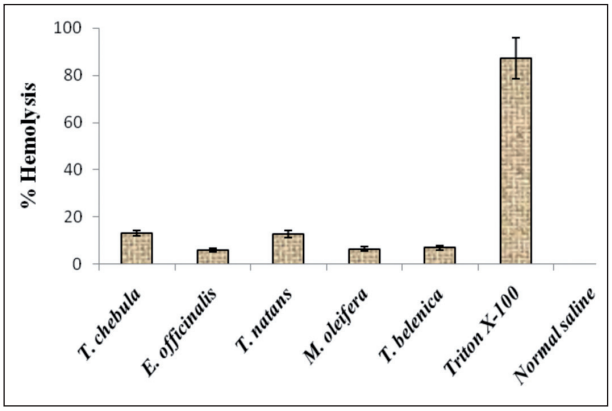

Figure 5. Cytotoxic (% hemolysis) activity of methanolic extract of selected medicinal plants.

Cytotoxic potential by hemolytic assay

In vitro cytotoxic potential of methanolic extract of selected medicinal plants was determined through hemolytic assay against the normal human erythrocytes in current study. The cytotoxic potential of selected medicinal plants methanolic extracts through hemolytic assay was expressed in terms of percentage hemolysis of erythrocytes and stated as mean±standard deviation of three replicates. All the tested plants extract exhibited very low percentage hemolysis of human erythrocytes. The lowest cytotoxic activity was exhibited by methanolic extract of *E. officinalis* with percent hemolysis of human erythrocytes in terms of mean±SD values of 5.89±0.78 % among the tested medicinal plants methanolic extract. Highest percent hemolysis was shown by methanolic extract of *T. chebula* with an average±SD values of 13.12±1.2 % followed by methanolic extract of *T. natans, T. belenica* and *M. oleifera* with an average±SD values of 12.8±1.4%, 6.92±0.94% and 6.45±0.86% respectively. Triton X-100 showed 87.3±8.64 % hemolysis which was used as positive control while normal saline was used as negative control exhibiting 0% hemolysis (Figure 5).

Conclusion

The study concluded that medicinal plants have very strong antioxidant activity as seen in different methods. These medicinal plants contain active ingredients like flavonoids and phenolics that exhibit antioxidant activity. Furthermore, isolation and characterization needed that these medicinal plants may contain novel active compounds which may be isolated. Medicinal plants extract having useful bioactive compounds can be further characterized and the research can be linked to industrial level to explore different biological activities so that these useful medicinal plants can be brought under the use of pharmaceutical industries.

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Correspondence:
Dr. Muhammad Riaz
Department of Allied Health Sciences,
Sargodha Medical College, University of Sargodha,
Sargodha-Pakistan
E-mail: riazmlt786@gmail.com