In vitro antioxidant activity of methanolic extracts of various parts of *Leptadenia pyrotechnica* (Forssk.) Decne.

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Abstract: *Leptadenia pyrotechnica* is a desert plant and its unripe fruits are cooked as a vegetable. Besides, this plant is also used for treating various ailments by the dwellers, yet very little is known about its free radical scavenging activity. Methanolic extracts of aerial parts and roots of this plant were evaluated for their free radical scavenging activity through 2,2' diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) scavenging, hydrogen peroxide scavenging and reducing power assays. Results revealed that there is strong free-radical scavenging activity lying in both parts comparable with synthetic antioxidant i.e. Butylated Hydroxy Anisole (BHA). The activity was found increased in a concentration-dependent manner. Root extracts showed significant DPPH$^{\bullet}$ and hydrogen peroxide scavenging activity, whereas highest electron donating capacity was observed in aerial parts extracts (O.D. of 2.38) at a concentration of 100µg/mL. This research work will be helpful in the discovery of novel antioxidants from *L. pyrotechnica* that may replace synthetic antioxidants.

Keywords: *Leptadenia pyrotechnica*, free-radical scavenging activity, flavonoids, plant parts, extraction.

INTRODUCTION

Various types of endogenous (metabolic reactions) and exogenous sources (food and pollution etc.) introduce diverse types of 'oxidant' and 'antioxidant' species in human bodies. Reactive Oxygen Species (ROS) such as super oxide anion, hydroxyl radicals and hydrogen peroxide are example of oxidant species (Ebrahimzadeh *et al.*, 2010). At lower concentrations, ROS have positive effects but at higher concentrations they have a potential to react with any cellular component like DNA, RNA, proteins and lipids thus leading to deleterious effects (Pham-Huy *et al.*, 2008). On the other hand, antioxidants can scavenge such species, detoxify and protect bodies from damage (Dharmishtha *et al.*, 2010).

Oxidative stress is developed when generation of oxidants increases as compared with antioxidant species in body. This stress culminates in a multitude of diverse diseases and complexities including cancer, aging, cataract, autoimmune disorders, arthritis, cardiovascular and neurodegenerative diseases (Behl and Mosmann, 2002; Finkel and Holbrook, 2000; Willcox, *et al.*, 2004; Pham-Huy *et al.*, 2008). Synthetic antioxidants (e.g. BHA and BHT) have serious risks associated with them as these can generate chain reactions leading to synthesis of more free radicals, leading to carcinogenesis (Ito *et al.*, 1983). This situation calls for search of alternate sources of antioxidants. Nowadays, researchers have started focusing on search of 'complementary and alternative medicine' (Wang *et al.*, 2003). Antioxidants from plant-base are one of such reliable sources as these are a part of our diet and are more compatible with the prevailing biological environments.

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Role of antioxidants-rich dietary supplements is appreciable and is known to enhance the potential of chemotherapy (Conklin, 2000). Fruits, vegetables and other dietary components may play potential role in protecting and preventing development of diseases in cases of oxidative stress but once a disease has developed, it needs treatment, which may possibly be achieved through antioxidant drugs developed from plant origin. Researchers are now paying heed toward search of novel antioxidants from plants for the last couple of years.

Leptadenia pyrotechnica (Forssk.) Decne. commonly known as Khip or khimp is a medicinal plant, native to hot deserts of Pakistan, India, Arabia, Egypt, Somalia, Chad, Libya and Algeria (Qureshi *et al.*, 2012). This is a leafless and much branched shrub, belonging to family Asclepiadaceae of plants, unripe pods/aerial parts of which are cooked as a delicious vegetable (Munazir *et al.*, 2010). This plant having an extensive root system, acts as soil binder and is known as Khimp in India and Khip or Barda in Pakistan (Qureshi *et al.*, 2012). Although, there are few records on phytochemical investigation of this plant species, but there is no comprehensive report on assessment of its potential antioxidant activity so far. The present study was undertaken to determine antioxidant activity of methanolic extracts of roots and aerial parts of this valuable medicinal plant.

MATERIALS AND METHODS

Preparation of extracts

Plant samples of *L. pyrotechnica* were collected from Thal desert of Pakistan. Methanolic extracts were prepared through maceration technique, using a slightly

modified method described by Walia *et al.* (2011). In this method, methanol (300 mL) was added to 200g of powdered samples of both plant parts and shook constantly at 200 rpm for 24 hours at 28°C. The mixtures were centrifuged at 8000 rpm for 15 minutes and filtered using Whatman filter paper 40. The residual powder was soaked in fresh solvent, agitated violently for 2-3 minutes and filtered again. This process was repeated three to four times for complete extraction of phytochemicals from the powdered samples. The supernatants, thus obtained were combined from each sample and dried under sterilized conditions in hot air oven maintained at 40°C. The resultant sticky gummy mass was stored at 4°C for further use. After that, varying concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/mL) of resultant extracts of aerial parts and roots and Butylated Hydroxy Anisole (BHA) were prepared in methanol.

Determination of free radical scavenging activity:
DPPH$^{\bullet}$ ***stable free radical scavenging assay***
Method of Choi *et al*., (2002) was used for this purpose. To every 2.5 mL of sample (i.e. dilution of plant extracts and standards), a total of 1 mL of DPPH in methanol (0.3 mM) was added and left at room temperature to react for half an hour. After that, absorbance of each reaction mixture was observed at 518 nm. DPPH in methanol was taken as blank. Each sample was replicated thrice and values recorded spectrophotometrically were used to calculate percent activity.

Hydrogen peroxide scavenging assay
Method of Ebrahimzadeh *el al.* (2010) was used Space please for assessment of free radicals generated during hydrogen peroxide assay. 40 mM solution of H_2O_2 prepared in phosphate buffer solution was mixed with all concentrations of each test sample and incubated for 10 min. Absorbance of reaction mixtures was measured at 230nm against a blank solution. Mixture of methanol and hydrogen peroxide was taken as blank. Results were expressed as percent scavenging activity of free radicals.

Reducing power assay
The reducing power was determined by using slightly modified method of (Ebrahimzadeh *el al.*, 2010). Each methanolic sample extract (2.5 ml) was mixed with 2.5 ml. Sodium Phosphate Buffer (0.2 M, pH 6.6) and 2.5 ml 1% Potassium ferricyanide $\{K_3 Fe (CN)_6\}$ and incubated for 20 min at 50°C. Trichloroacetic acid (10%, 2.5 mL) was added to the resultant mixture so as to stop the reaction. This mixture was then centrifuged at 10,000 rpm for 8 min and upper layer (2.5 mL) was mixed with equal quantity of deionized water (i.e. 2.5 mL). Reducing power of standard and plant extracts was measured spectrophotometrically at 700 nm after addition of 0.5 mL of 0.1% ferric chloride.

STATISTICAL ANALYSIS

In case of DPPH$^{\bullet}$ scavenging activity and H_2O_2 scavenging assays, % scavenging activity (S.A.) was calculated by using the following formula,

$$\text{S.A. (\%)} = \frac{\text{As-Ac}}{\text{Ac}} \times 100$$

S.A. stands for Scavenging Activity, As for absorbance of sample and Ac for absorbance of control

Average and standard deviation of percent free radical scavenging activity was calculated for all three replica in each assay. Regression models were built for average values of DPPH and H_2O_2 scavenging activities, average percentages were plotted against respective concentrations for each plant part/standard. IC_{50} values were calculated by using regression equations generated through scatter plot analysis, where IC_{50} represents concentration of a sample required to bring about 50% free radical scavenging activity. Values of regression coefficients (R^2) were observed to describe strength of dependent and independent variables. SC_{50} values were determined from average values, where SC_{50} denotes half of a maximum scavenging activity of a sample. Two-way Analysis of Variance (ANOVA) was applied to investigate the effect of plant parts and concentrations and interaction between these two independent variables. Total flavonoids content and total tannins content was expressed as average and standard deviation and correlation with IC_{50} were determined.

RESULTS

Both plants parts of *L. pytotechnica* exhibited promising free radical scavenging activity, even in minute concentrations tested *in vitro.* Comparison of percent scavenging activities reflected that root extracts showed higher activity than aerial parts extracts in case of DPPH$^{\bullet}$ and hydrogen peroxide scavenging assays, while in reducing power assay, this trend continued up to concentration of 40 µg/mL and then activity of aerial parts extracts became higher than roots extracts (Figures 1, 2 and 3). Moreover, there was highly significant difference among all tested concentrations ($p<0.01$) and interestingly significant interaction was observed between plant parts and concentrations ($p<0.01$).

DPPH* free radical scavenging assay
Table 1 represents the results of regression analysis in case of DPPH$^{\bullet}$ scavenging assay for both plant parts and BHA (including values of slope, intercept and regression coefficients along-with respective IC_{50} and SC_{50} values), whereas table 2 represents the results of all these parameters in case of H_2O_2 scavenging assay. Strong correlation (in terms of regression coefficient, R^2) was observed between all tested concentrations of extracts and their respective scavenging activities. It was also observed

that the results of free radical scavenging activity increased in a concentration-dependent manner (figs. 1, 2 and 3). In the present study, total flavonoids and tannins contents were determined (tables 3 and 4). Correlation analysis was carried out to discover the relationship between flavonoid and tannin contents in both plant parts of *L. pyrotechnica* and their free radical scavenging activities (i.e. proton donating and electron donating capacities). A very strong positive correlation was observed between total flavonoids content and antioxidant activity (the extent of correlation was +1).

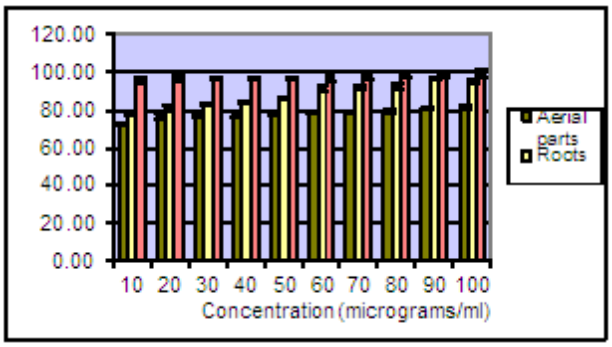

Fig. 1: DPPH scavenging assay of roots and aerial parts of *Leptadenia pyrotechnica.*

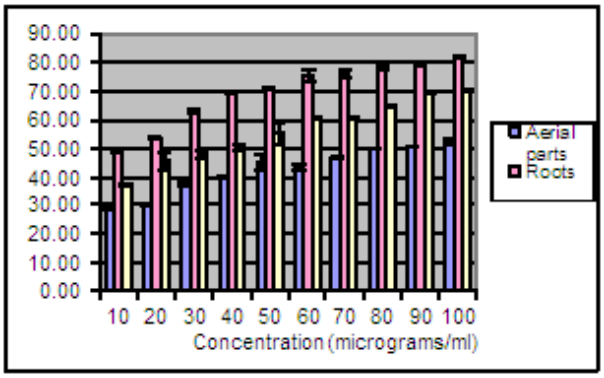

Fig. 2: Hydrogen peroxide scavenging assay of roots and aerial parts of *L. pyrotechnica*.

It was observed that the value of slope of regression line was the lowest in case of BHA (0.0319) and highest in case of aerial parts (3.2932), which reflected that with a unit change in concentration, percent activity changed remarkably in aerial parts while BHA responded the least. Whereas, value of intercept was the highest in case of BHA (95.934) and the lowest in aerial parts (65.85), where intercept represents the average change in dependent variable (percent scavenging activity) when the value of independent variable is zero (i.e. even though the value of concentrations is zero, the regression model shows that there is some activity, which is highest in case of BHA and lowest in case of aerial parts). IC_{50} value of BHA was lowest, thus reflecting a strong free radical scavenging activity. Whereas, IC_{50} value of roots and aerial parts were also remarkably high (i.e. -128.146 and 0.6824 µg/ml respectively) reflecting very high antioxidant activity. SC_{50} was highest in case of BHA, followed by root extracts and aerial parts (i.e. 49.575, 48.405 and 40.715µg/mL respectively).

Hydrogen peroxide scavenging assay

Comparison of percent scavenging activity of *L. pyrotechnica* tested through hydrogen peroxide scavenging assay showed that there was highly significant difference in activity ($p<0.01$). The root extracts showed promising scavenging activity at all tested concentrations ranging from 10µg/ml to 100µg/ml (fig. 2). This activity was higher than BHA, which is a synthetic antioxidant product. Aerial parts extracts showed less scavenging effect at all concentrations. These differences in activity of plant parts may be attributed to the differences in phytochemical profile (especially that of phenolic compounds). Very high values of regression coefficient were observed (i.e. R^2 of 0.9488 in case of aerial parts and 0.9785 in roots). These values are depictive of strength of relationship between dependent and independent variables, where scavenging activity is dependent variable and concentration of extract is independent variable. Here, a highly significant difference in activity of all tested concentrations was observed ($p<0.01$) and the activity increased with increase in concentration of extract and hence a strong regression coefficient was observed. A highly significant interaction between plant parts and concentrations was shown by ANOVA with $p<0.01$.

Higher values of slope in case of root extracts (i.e. 14.943) followed by that of aerial parts (11.227) were observed. BHA showed the lowest value of slope (0.3493), which reflects that the free radical scavenging activity of BHA was less affected by increasing concentration. IC_{50} value for roots was the lowest (i.e. 0.395µg/ml), followed by aerial parts (0.651µg/ml). BHA exhibited highest IC_{50} value (i.e. 36.86µg/ml). SC_{50} value for roots was the highest (i.e. 40.955 µg/ml), followed by aerial parts (26.315µg/ml) that was lower than that of BHA (35.095µg/ml).

Reducing power assay

Both plant parts showed significant reducing power, even higher than that of BHA (fig. 3). Reducing power of the extracts is attributed to the presence of some components in the crude extracts that have electron donating capacity thus reducing Fe^{+3} into Fe^{+2}. It was interesting to note that there was a gradual increase in the reducing power in such a way that reducing power of aerial parts exceeded roots and BHA extracts at higher concentrations. ANOVA also showed that there was highly significant difference in free radical S.A. of both plant parts ($p<0.01$), same was the case with all concentrations and there was a highly significant interaction between plant parts and concentrations. Highest electron donating capacity was observed in aerial parts extracts (O.D. of 2.38) at a concentration of 100µg/mL.

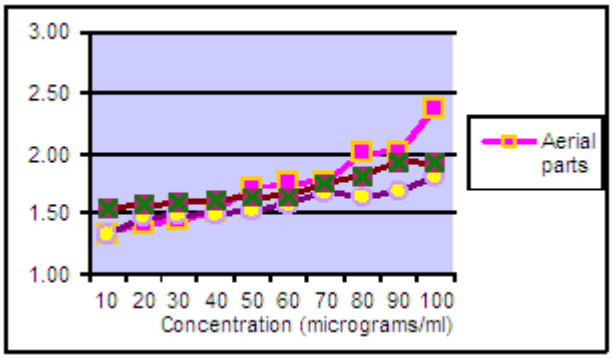

Fig. 3: Reducing power assay of aerial parts and roots of *L. pyrotechnica*.

Table 2: H_2O_2 Scavenging Assay, regression equation, regression coefficient and IC_{50} for roots, aerial parts and BHA

Parts	R.E. (y=ax+b)	R^2	IC_{50} (µg/ml)	SC_{50} (µg/ml)
Aerial parts	Y=11.227Ln(x)-0.2196	0.9488	0.651	26.315
Roots	Y=14.943Ln(x)+12.87	0.9785	0.394	40.955
BHA	Y=0.3493x+37.125	0.9766	36.859	35.095

DISCUSSION

Free radical scavenging capacity of plants is one of the possible mechanisms of action of plant-based drugs. Value of natural resources is being acknowledged worldwide which has resultantly compelled researchers across the globe for experimentation in the surge for natural pharmaceutical components (Okeri and Alonge, 2006). Pakistan is quite rich in unique treasure of medicinal plants that can be utilized for development of precious medicines, which may in turn strengthen pharmaceutical industry of this country (Rasul *et al.*, 1989). Keeping the importance of such medicinally active plants, there is an urgent need to evaluate the indigenous flora for therapeutic impacts. *L. pyrotechnica* is a medicinally active plant that is used by local residents of deserts in Pakistan for treatment of various ailments including inflammation. The present study was designed to investigate extracts of roots and aerial parts of *L. pyrotechnica* for free-radical scavenging activity.

DPPH scavenging assay is a convenient and easy to use rapid method that is widely appreciated for its potential in evaluation of antioxidant activity of plants (Nickavar *et al.*, 2007). In the present study, antioxidant potential of *L. pyrotechnica* was tested by employing the oxidation-reduction properties of DPPH free radical. It was observed that the root extracts showed higher percent scavenging activity, which may rightly be attributed to the proton donating capacity of some components in the crude extracts under investigation. Aerial parts extracts also showed higher proton donating capacities and hence free radical scavenging capacity (almost 80% S.A. at all tested concentrations of crude extracts).

Table 1: DPPH assay, regression equation, regression coefficient and IC_{50} for roots, aerial parts and BHA

Parts	R.E. (y=ax+b)	R^2	IC_{50} (µg/ml)	SC_{50} (µg/ml)
Aerial parts	Y=3.2932Ln(x)+65.85	0.8108	0.6824	40.715
Roots	Y=0.2082x+76.68	0.9617	-128.269	48.405
BHA	Y=0.0319x+95.934	0.874	-1439.94	49.575

A highly significant difference in activity of all tested concentrations was observed and DPPH∗ free radical scavenging activity was found to be concentration dependent as it continued to increase with increasing concentrations (Meena *et al.*, 2012; Malathi *et al.*, 2012). This may be due to the increase in concentration of compound or group of compounds which have capacity to donate protons or electrons, thus neutralizing $DPPH^*$ (depicted by color changes from bluish to yellowish). Interestingly, the activities of plant extracts were quite high even in very minute concentrations and are comparable with a purified antioxidant substance i.e. BHA, although the extracts are crude and contain trace amounts of antioxidant compounds. A highly significant difference was observed in activity of both plant parts ($p<0.01$) and strong interaction between plant parts and all tested concentrations ($p<0.01$) was depicted by Two-Way ANOVA.

$DPPH^*$ free radical scavenging capacity of this plant is higher than many plant species reported by Kshirsagar and Upadhyay (2009) including *Litsea glutinosa* (90.57%), *Grewia sapida* (95.46%), *Saraca asoca* (95.52%), *Syzygium cerasoides* (93.60%), *Pterospermum semisagittatum* (96.99%), *Wendlandia wallichii* (96.99%), *Alocasia fornicata* (41.06%), *Alpinia malaccensis* (21.63%), *Callicarpa arborea* (53.65%), *Cassia nodosa* (78.96%). These results emphasized that the extracts from *L. pyrotechnica* may be a potential source of antioxidant compounds if investigated further for purification, identification and characterization of antioxidant compounds for drug development in future.

CONCLUSION

The methanolic extracts of both plant parts of *L. pyrotechnica* possessed significant free radical scavenging activity which was comparable to a synthetic antioxidant compound (i.e. BHA). Based on results it can be concluded that the extracts may prove effective in treating various diseases that are caused by free radicals produced in body as a result of extreme oxidative stress, thus can provide an alternative source of various synthetic antioxidant agents e.g. BHA. However, before its application, in-depth studies (for instance *In vivo* assays)

may be conducted in future to see the potential toxicity of this plant.

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