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RESEARCH ARTICLE

Evaluation of free radical scavenging and antioxidant potential of *Melilotusparvifolra*

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Manuscript Info *Abstract*

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In this paper the % scavenging of free radical by *Melilotusparvifolrahas* been presented. The obtained methanolic extract of *Melilotusparvifolra*was evaluated for2.2 diphenyl 1.1- picrylhydrazlhydrazyl (DPPH) radical scavenging activity, 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) radical scavenging activity, superoxide radical scavenging activity, hydrogen peroxide scavenging activity and hydroxyl radical scavenging activity. Total phenolic and flavonoids contentswere also measured. The results show that methanolic extract of *Melilotusparvifolra*has good scavenging ability except ABTS radical scavenging activity. The total phenolic and flavonoids content were calculated as 0.35mg/g and 0.07 mg/g of extract respectively

1. Introduction;

The oxidative stress refers as the imbalance between the productions of free radicals of oxygen species, such as super oxide (O_2), hydroxide radical, nitric oxide radicalsand antioxidant defense system. These radicals are produced due to the excessive metabolism inside a biological system while antioxidant enzymatic and non enzymatic system having the ability to detoxify the reactive intermediates or having the ability to repair the resulting damage. The turbulence in standard redox state causes the toxic effects as well as the fabrication of peroxides and free radicals [1]. These causes the cell components damage which included proteins, lipids and DNA. The oxidative stress can cause various diseases including cancer and Alzheimer's and also it is related to the body aging process [2]. These may also react with radical's i.e.O_2 which producesperoxynitrite ($ONOO^-$) a best oxidant [3]. The epidemiological as well as laboratory studies have shown that some of the edible plants entirely or some of the ingredients having the antioxidant properties [4].Khan et al. [4] and Sahreen et al. [5] reported about the free radical scavenging properties of some medicinal plants.Mellitus species are monocarp, annual or biennial herbs which are belonging to the legume family (Leguminosae). Melilotusparvifolra is an erect much-branched slender glabrous annual herb having 1–2 ft height. Petioles have 12–18 lines; leaflets are obovate-cuneate, 6–9 lines long and finely toothed. Flowers are short-stalked, auxiliary racemes pedicel scarcely, corolla is pale yellow and twice the calyx [6]. Mellilotusparvifolra is distinguished from alfalfa by the absence of pubescence of thinner side leaves and bitter taste [7]. This paper presents the free radical scavenging properties of *Melilotusparvifolra*. The scavenging properties were measuredin term of2.2 diphenyl 1.1- picrylhydrazlhydrazyl (DPPH) radical scavenging activity, 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) radical scavenging activity, superoxide radical scavenging activity, hydrogen peroxide scavenging activityand hydroxyl radical scavenging activity as well as total phenolic and flavonoids contentswere measured.

2. Materials and method

2.1. Plant Collection:

Meliltousparviflora was collected from District Bannu during the June 2012 and was identified by Prof. AbdurRehman GPGC Bannu. Collected plant samples were dried under the shadow at a room temperature for 40 days then grinded it in to the fine powder.

2.2. Plant Extraction:

Nearly 1400 grams of the plant fine powder of *Meliltousparviflora* were socked in 6 liters of the 80% methanol with gentle shaking, brought from the musajee science trade Peshawar placed it at room temperature for 7 days.After the seven days the plant is extracted and filtered by using whatmman filter paper and concentrated with the help of the rotary evaporator after the concentration, the extra methanol was evaporated at 37 ºC to obtain crude extract.

2.3. Antioxidant activity bioassay;

2.3.1. DPPH Radical Scavenging activity

The DPPH assay was done according to the modified method of Khan et al. [8]. The fresh stock solution was prepared by dissolving 2.4 mg DPPH with 100 ml methanol and then stored at 20 °C. The working solution was obtained by diluting DPPH solution with methanol to obtain an absorbance of about 0.980 (± 0.02) at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the fractions at varying concentrations (1-100 µg/ml in respective solvent). The solution in the test tubes were shaken well and incubated in the dark for 30 min at room temperature. Then the absorbance was taken at 517 nm. The percentage of DPPH radical scavenged as the following equation:

Scavenging effect (%) = [(control absorbance-sample absorbance) / (control absorbance)] ×100.

2.3.2. ABTS radical scavenging activity

With certain alterations the ABTS radical scavenging activity was measured using the method developed by Sahreen et al. [9]. Equal volumes of 7 mM ABTS solution and 2.45 mM potassium per sulfate solution were mixed to prepare stock solution and incubated in the dark for 12 h at room temperature to yield a dark-colored solution consisting of $ABTS^{\bullet+}$ radicals. 50% methanol and stock solution were mixed to prepare working solution for an initial absorbance of about 0.700 (± 0.02) at 745 nm, with control temperature set at 30°C. Free radical scavenging activity was determined by mixing 300 µl of different concentrations (25-250 µg/ml in methanol) of methanolic extract with 3.0 ml of ABTS working standard. When the solutions were mixed then exactly after 1 min and 6 min of the decrease in absorbance was measured. Experiment was done in triplicate. Ascorbic acid was used as positive controls in this experiment. The scavenging activity was determined based on the percentage of ABTS radicals scavenged by the formula given below:

$$\%\text{scavenging}=[(A0-As)/A0]\times 100$$

Where A_0 is absorption of control, A_S is absorption of sample solution.

2.3.3. Determination of superoxide radical scavenging activity

The modified protocol of Ahmed et al [10] Was used to determined superoxide radical scavenging activityreaction mixture was prepared by mixing 1 ml of nitrobluetetrazolium (NBT) solution (l M NBT in 100 mM phosphate buffer, pH 7.4), 0.1 ml of different fractions and ascorbic acid (50 mM phosphate buffer, pH 7.4) and 1 ml NADH solution (l M NADH in 100 mM phosphate buffer, pH 7.4). 100 µl of (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) was added in the mixture and the reaction was started. All The tubes were illuminated uniformly with an incandescent visible light for 15 min and before and after the illumination the optical density was measured at 530 nm. The percentage inhibition of superoxide production was assessed by comparing the absorbance values of the control and experimental tubes. Superoxide radical scavenging ability was determined by following formula:

$$\%\text{scavenging}= (1-Ae/A_o)\times 100$$

Where Ae is absorbance with sample and A_o is the absorbance without sample,.

2.3.4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assessed using the method developed by Sahreen et al [11] with some modifications. 2 mM solution of hydrogen peroxide was prepared in 50 mM phosphate buffer having pH 7.4. The concentration of hydrogen peroxide was determined spectrophotometrically at 230 nm using the molar extinction coefficient for H_2O_2 of 81 $mol^{-1}cm^{-1}$. 0.1 ml of various fractions (25-250 µg/ml in methanol), ascorbic acid was transferred into the test tubes and 50 mM phosphate buffer (pH 7.4) or solvent (methanol) was added to make their volumes up to 0.4 ml. 0.6 ml hydrogen peroxide solution was added and then all the tubes were vortexed and absorbance of the hydrogen peroxide was determined at 230 nm after 10 min, against a blank. 50 mM phosphate buffers without hydrogen peroxide were used as blank. Hydrogen peroxide scavenging ability was calculated by the following formula:

%scavenging= (1−Ae/Ao)×100
Where Ae is absorbance with sample and Ao is the absorbance without sample

2.3.5. Hydroxyl radical scavenging activity

With some modifications the Hydroxyl radical scavenging activity was assessed [12]. 2-Deoxyribose was degraded on exposure to OH radicals were generated by Fenton's reaction. Each fraction and ascorbic acid solution was prepared in methanol. The reaction mixture contained 0.45 ml of 0.2 molar sodium phosphate buffer (pH 7.0), 150 µl of 10 mM $FeSO_4$-EDTA, 150 µl of 10 mM 2-deoxyribose, 525 µl of H_2O, 150 µl of 10 mM H_2O_2, and 75 µl of sample solution (0.050-0.250 mg/ml in methanol). The reaction was started when H_2O_2 was added. After 4 hours incubation at 37°C, 750 µl of 2.8% trichloroacetic acid and 750 µl of 1% TBA in 50 mMNaOH was added and the reaction was stopped. Then for 10 minutes the solution was boiled, and then cooled in water. The absorbance of the solution was measured at 520 nm. Ascorbic acid (50-250 µg/ml) was used as a positive control. Hydrogen peroxide scavenging ability was calculated by the following formula:

%Superoxide radical scavenging activity=(1−absorbanceofsample/absorbance of control)×100

2.3.6. Total phenolic contents

With certain modifications the total phenolic content was assessed using the method developed by [13]. Methanolic solution of gallic acid was mixed (1 ml; 0.1-1.00 mg/ml) with 5 ml folin-Ciocalteu reagent (diluted tenfold) and Calibration curve was prepared. Before The addition of sodium carbonate (4 ml, 0.115 mg/ml) the mixture was incubated at room temperature for 5 min, and absorbance was measured at 765 nm. 1 ml of plant extracts dissolved in ethanol (1 ml; 0.1-1.00 mg/ml) was mixed with the reagents above, and after 120 minutes the absorbance was measured at 765 nm to determine total phenolic contents. Experiment was done in triplicate. The total phenolic contents in the extract were expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

2.3.7. Determination of the total flavonoids content;

The total flavonoids was assessed using the method developed by [13] with modifications. 250 µl of plant extract (5 mg/ml; dissolved in methanol) and rutin standard solution (15-240 µg/ml) was mixed with 1250 µl of distilled water in a test tube, and then 75 µl of a 5% (w/v) sodium nitrite solution was added. After 6 minutes 150 µl of (10% w/v) aluminum chloride solution was also added, and the mixture was allowed to stand at room temperature for 5 minutes before the addition of 0.5 ml of 1 mNaOH. The mixture was made up to 2500 µl with distilled water and mixed well and the absorbance was measured immediately at 510 nm.The result of sample was expressed as mg of rutin equivalents of total extractable compounds. Experiment was done in triplicate.

3. Result and Discussion

3.1. DPPH Radical Scavenging activity:

DPPH (1-1 diphenyl 1-2 picryl- hydroxyl) it is a free radical and has strong attracting ability from antioxidant [14]. The scavenging activity of the methanolic extract of *Meliltousparviflora* as shown in Fig 1.The graph shows that the concentration of methonlic extract is directly proportional to the %Scavenging of *Meliltousparviflora*methanolic extract.The minimum scavenging activity is shown by the lowest

concentration of 50 µg/ml thus the order of the scavenging activity is 50 µg/ml <100 µg/ml< 150 µg/ml <200µg/ml<250 µg/ml and as well as the ascorbic acid is used as a control. The result obtained by Falleh et al. [15] also similar to that of the present work. The presence of phenolandpolyphenoliccomponents are significantly inhibited the free radicals that cause oxidative stress.

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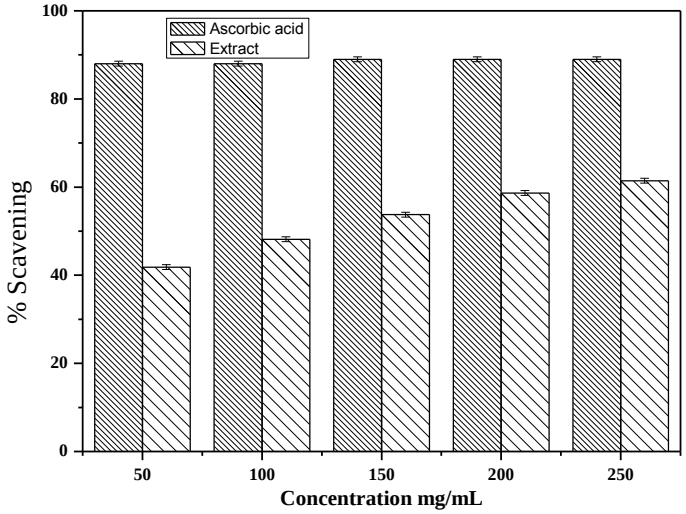

Figure 1. DPPH free radical scavenging of Melilotusparvifloramethanolic extract and ascorbic acid

3.2. ABTS ScavengingAssay:

2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (biochemical reagent)is a free radical source and has strong attracting ability from antioxidant.The scavenging activity of the methanolic extract of *Meliltousparviflora*is shown in Fig.2. The graph shows that as the concentration of methanolic extract increased the % scavenging of *Meliltousparviflora* extract was observed to increase. The minimum scavenging activity is shown by the lowest concentration of 50 µg/ml. The order of the scavenging activity for methanolic extract is 50 µg/ml <100 µg/ml< 150 µg/ml <200 µg/ml<250 µg/ml and as well as the ascorbic acid is used as a control.

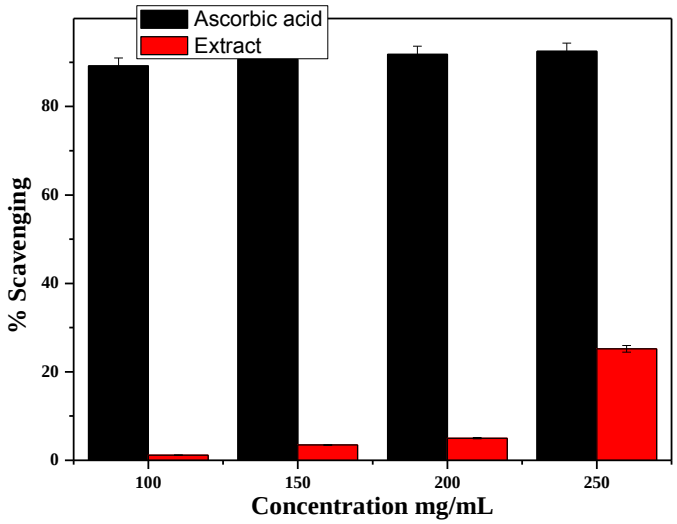

Figure 2. ABTS free radical scavenging of *Melilotusparviflora*methanolic extract and ascorbic acid

3.3. Scavenging activity of superoxide radical:

Superoxide radical are the reactive oxygen species (ROS), cause very harmful and toxic effects to cellular components, as well as contributing in many fetal diseases. The Fig. 3 shows scavenging of the various fractions of *Meliltousparviflora*methanolic extract possessed the most potent superoxide radical scavenging activity (72.4±3.2µg/ml) showed the scavenging effect near to standard compounds.

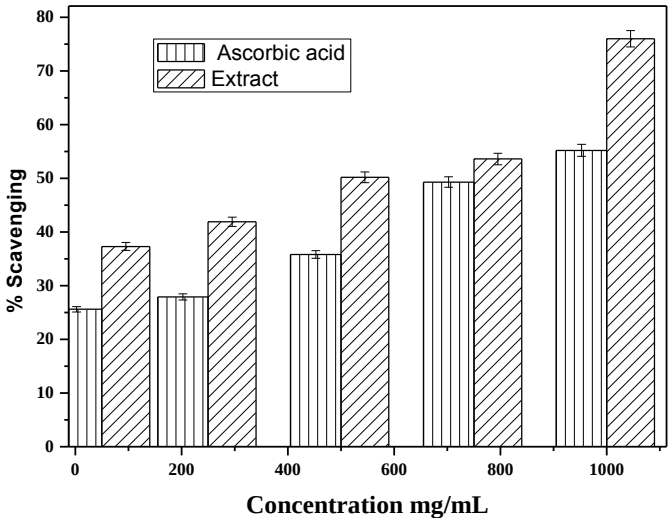

Figure 3. $^{-}O_2$ free radical scavenging of Meliltousparviflora extract and ascorbic acid

3.4. Hydrogen peroxide-scavenging activity:

The hydroxyl radical (•OH) negotiate membranes of cell at particular locations; react with most biologically active macromolecules resulting in tissue damage and death of the cell. Therefore, scavenging of •OH is necessary to protect the living systems. Fig.4shows the % inhibition of *Meliltousparviflora*.The Figure showed pronounced scavenging activity, markedly scavenged the free radial and was the most potent than reference chemical compound.

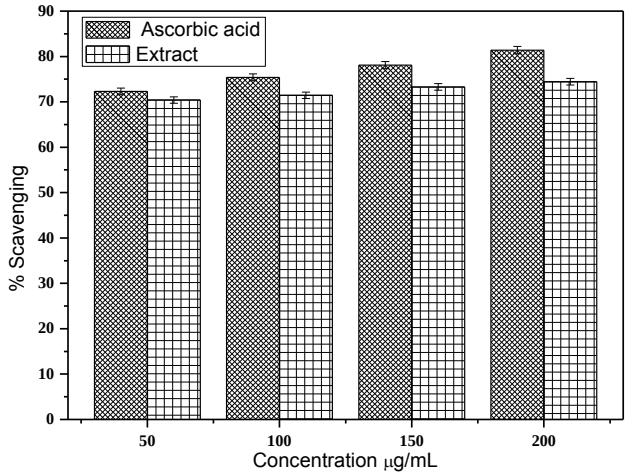

Figure 4. H_2O_2 free radical scavenging of *Melilotusparviflora*methanolic extract and ascorbic acid

3.5. Total flavonoids and phenolic contents:

The phenolic compounds determination was carried out using the Folin–Ciocalteau assay. Figure 5 shows total flavonoids and phenolic content of methanolic extract. Total phenolic contents were relatively high in concentration as compared to flavonoids content i.e. 0.35 and 0.07 mg/g extract respectively.

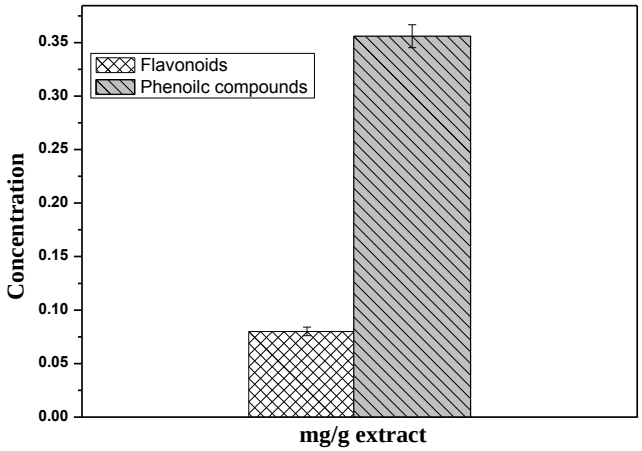

Figure 5. Total flavonoids and phenolic compounds in plant extract of Meliltousparviflora

4. Conclusion;

The methanolic extract of *Meliltousparviflora* evaluated for antioxidant properties was identified as a potential medicinal plant for scavenging the free radicals. The scavenging ability of methanolic extract was measured to increase with increase in their concentrations. The methanolic extract was noted to have low activity of scavenging against ABTS based free radicals. Total flavonoids and phenolic compounds that have been reported for antioxidant activity was measured in the methnaolic extract of *Meliltousparviflora*.

5. Acknowledgement

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