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Effect of pH on Antioxidant and Phytochemical Activities of Mulhatti Roots (*Glycyrrhiza glabra* L.)

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Abstract: *Glycyrrhiza glabra* L. is the most widely used herb in the ancient history of Ayurvedic medicine, as a medicinal value as well as an aromatic herb, and it is commonly known as Mulhatti. Mulhatti roots are useful for medically and are also a good source of phytoproducts and secondary metabolites present in Mulhatti roots are triterpenoid saponin, glycosides, glycyrrhizin, prenylated biaurone, licoaagrone, 7-acetoxy-2-methylisoflavone, 4-methylcoumarin, liqcoumarin, glycyrrhetinic acid, quercetin, liquiritigenin, isoliquiritigenin, etc. This study was carried out to study the evaluation of phenolic compounds, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity and general antioxidant capacity of water extracts of Mulhatti roots prepared at different pH values, namely 2, 4, 7 and 9. Data have shown great differences in terms of results. Most of the phenolic compounds are at pH 7 (19.25), followed by pH 9 (17.25), pH 2 (14.62) and pH 4 (8.89 mg GAE/g), respectively. Similarly, the flavonoid data also showed variations, the maximum has been present in pH 2 (5.39), then pH 7 (3.02), pH 9 (1.79) and pH 4 (1.40 mg CE/g), respectively. The value for DPPH IC_{50} free radical scavenging activity was the lowest at pH 7 (82.22), followed by pH 2 (110.40), pH 4 (111.99) and pH 9 (146.24 μg/mL) and IC_{50} reference standard (ascorbic acid) was 59.52 μg/mL in distilled water. The total capacity of the antioxidant was the highest at pH 2 (9.93) followed by pH 4 (5.54), pH 7 (5.34) and pH 9 (4.23 mg AAE/g). According to current research, pH 7 is the best for phytochemicals as well as antioxidants that catch harmful radicals.

Key words: Mulhatti (*Glycyrrhiza glabra* L.) phytochemicals, secondary metabolites, phenolics, flavonoids, DPPH free radical scavenging activity, total antioxidant capacity.

1. Introduction

Medicinal plants have been common since the origin of the earth, which ancient people used efficiently as a remedy for various diseases [1]. The native medicinal plants and herbal medicines are the potential source of alternative medicine and are used extensively to treat various health ailments [2]. *Glycyrrhiza glabra* L. is one of the most widely used herbs from the ancient medical history of Ayurveda, both as a medicine and as an aromatic herb [3]. It is commonly known as Mulhatti (in Hindi), Sweetwood (in English), and Madhuka (in Sanskrit). It is a perennial herb that belongs to the Fabaceae family and is found primarily in the southern zone of Europe and also in some wild-growing species in parts of Asia [4]. Its roots are rich in minerals (Ca, K and Na), phenolics, alkaloids, carbohydrates, saponins, flavonoids, lipids, proteins and tannins [5] and the secondary metabolites present in Mulhatti are triterpenoid saponin, glycosides, glycyrrhizin, prenylated biaurone, licoaagrone, 7-acetoxy-2-methylisoflavone, 4-methylcoumarin, glycyrrhetinic acid, quercetin, liquiritigenin, isoliquiritigenin, etc. Antioxidant contained in plants scavenges harmful free radicals from body and these are the species that are capable of independent existence and contain one or more unpaired electrons that react with other molecules by accepting or giving up electrons [6]. Today, due to their carcinogenic properties, there is an increased interest in naturally occurring antioxidants for use in food or medical materials as a substitute for synthetic antioxidants [7]. Mulhatti (*G. glabra* L.) is also an excellent source of

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natural antioxidants. The objective was to study the effect of aqueous extracts of Mulhatti (*G. glabra* L.) roots with different pH values (2, 4, 7 and 9) on the total amount of phenols, flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity (free radical neutralization method) and total antioxidant capacity by modified phosphomolybdenum assay.

2. Materials and Methods

2.1 Plant Materials Collection

Mulhatti roots (*G. glabra* L.) were obtained in the Experimental Area of Medicinal, Aromatic and Potential Crops Section, Department of Genetics and Plant Breeding, Choudhary Charan Singh Agricultural University, Hisar, Haryana in 2017. Plant part photograph is shown in Fig. 1: in Fig. 1a, dried root and in Fig. 1b, powdered form of Mulhatti.

2.2 Preparation of Plant Extracts

Five grams (5 g) powdered samples of Mulhatti roots were packed in thimbles prepared from Whatman No. 1 filter paper and extraction was carried out using classical soxhlet apparatus. This apparatus fitted with 500 mL round bottom flask and distilled water as a solvent was added about half siphon (240-270 mL) of different pH values (2, 4, 7 and 9) and pH was adjusted using concentrated HCl and NaOH pellets. Then, extraction was carried out at the boiling point of water. The solvent vapours rose up in the column and condensed in the condenser part of the apparatus. After condensation, they flowed into the thimble of the chamber filled with samples of Mulhatti roots and each extract was filtered. This process was repeated three times. The resulting filtrate was stored in vials for further experiment.

2.3 Total Phenolic Content

The phenolic compounds present in roots of Mulhatti aqueous extracts at different pH values (2, 4, 7 and 9) were estimated by Folin-Ciocalteu method [8]. Estimate the total phenols in aqueous extracts of different pH values; 0.2 mL of each extract was diluted with the respective solvent to adjust the absorbance within the calibration limits. One milliliter (1 mL) of 1 mol/L Folin-Ciocalteu reagent was added and 2 mL of Na_2CO_3 (20%, *w*/*v*) was mixed and the final volume was made up to 10 mL with distilled water for 10 min at 6,000 rpm centrifuged. Similarly, a blank was prepared and at place of extracts respective solvent was used. At 730 nm, absorbance of the supernatant solution was measured against a blank prepared on ultraviolet-visible (UV-Vis) double beam spectrophotometer model UV 1900 (Shimadzu,

(a) (b)

Fig. 1 Dried roots (a) and powdered form of Mulhatti (b).

Japan). The amount of total phenolic compounds present in the various extracts was calculated from a standard curve and expressed in mg GAE/g.

2.4 Flavonoids Content

The flavonoids present in the roots of aqueous Mulhatti extracts at different pH values (2, 4, 7 and 9) were estimated by colorimetric aluminium chloride assay [9]. To estimate the total flavonoids in aqueous extracts with different pH values, 1 mL of each extract was placed in test tubes and 4 mL of distilled water, 0.3 mL of $NaNO_2$ (5%) and 0.3 mL of $AlCl_3$ (10%) were added after 5 min. Immediately 2 mL NaOH (1 M) were added. The final volume was made up to 10 mL with distilled water. A blank value was prepared in a similar manner but the corresponding solvent was used instead of extracts. After thoroughly shaking the solution, the absorbance at 510 nm was measured against a blank value which had been produced on a UV-Vis double beam spectrophotometer model UV 1900 (Shimadzu, Japan). The amount of flavonoids present in the various extracts was calculated using a standard curve and expressed in mg CE/g.

2.5 Antioxidant Activity

Antioxidant activity by DPPH free radical scavenging activity method [10]: aqueous extracts of roots of different pH values (2, 4, 7 and 9) of Mulhatti were taken and dried completely and the weights of their dry mass were noted. Dry mass of aqueous extracts was redissolved in an appropriate amount of 50% (*v*/*v*, methanol:water) since it was not soluble in pure methanol to obtain a stock solution of 1,000 μg/mL based on the dry weight of the extract. Different concentrations (5-500 μg/mL) were prepared from the stock solution 1,000 μg/mL by appropriate dilution with 50% (*v*/*v*) water:methanol. To assess antioxidant activity, 1 mL of extract/fraction of each concentration was taken into glass vials and added 2 mL of DPPH (0.1 mM in 100% methanol), covered with lids and mixed it well for 5 min. For an aqueous extract of different pH values, a stock solution of DPPH was prepared in 50% (*v*/*v*) methanol:water. A control was performed in place of the sample using 1 mL of each solvent. They were incubated for 30 min in the dark and after this absorption of the sample and the control were measured against a blank value which contained pure methanol at 517 nm UV-Vis double beam spectrophotometer model UV 1900 (Shimadzu, Japan) and each sample was promoted to three replications. Using Microsoft Excel software, a graph was drawn up in which the DPPH radical scavenger activity (%) was plotted on the y-axis versus the extract concentration (μg/mL) on the x-axis, then a quadratic regression equation ($y = ax^2 + bx + c$) was received. The obtained equation was converted in the form of ($ax^2 + bx + c = 0$) by putting $y = 50\%$. Using equation ($ax^2 + bx + c = 0$), the IC_{50} value was calculated by applying equation:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (μg/mL).

The percentage of DPPH radical scavenging activity (%DPPH*$_{SC}$) was calculated using:

$$\%\text{DPPH}^*_{SC} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

where, $A_{control}$ = absorbance of control, A_{sample} = absorbance of the sample.

2.6 Total Antioxidant Capacity

Estimate the total antioxidant capacity of aqueous extracts of Mulhatti roots of different pH values (2, 4, 7 and 9) by the modified phosphomolybdenum method [11]; 0.3 mL of each extract was taken into glass vials and 3 mL of phosphomolybdenum reagent was added and the solution was mixed well, covered with lids. They were incubated at 95 °C for 90 min. After this, the contents of vials allowed to cool down and absorbance was measured at 695 nm on UV-Vis

double beam spectrophotometer model UV 1900 (Shimadzu, Japan)

Table 1 Quadratic regression equations for IC_{50} (µg/mL) values of aqueous extracts of Mulhatti roots at different pH values (2, 4, 7 and 9).

pH	Mulhatti roots
2	$y = -0.0006x^2 + 0.4268x + 10.195$ $R^2 = 0.9717$
4	$y = -0.0006x^2 + 0.4639x + 5.5708$ $R^2 = 0.9932$
7	$y = -0.0013x^2 + 0.6439x + 5.8457$ $R^2 = 0.9896$
9	$y = -0.0004x^2 + 0.3594x + 5.9956$ $R^2 = 0.9717$

against a blank prepared. Similarly, a blank was prepared; at place of extract/fraction respective solvent was used. The total antioxidant capacity was calculated in aqueous extracts from the standard curve and expressed as mg AAE/g.

2.7 Statistical Analysis

For statistical study all results are calculated in triplicates and expressed in mean ± SD. One-way analysis of variance (ANOVA) was performed to assess any significant differences between sample means in online statistical analysis (OPSTAT). IC_{50} values of DPPH free radical scavenging activity were calculated using a quadratic regression equation given in Table 1. The correlation between total phenolic compounds, total flavonoids and IC_{50} values of DPPH free radical scavenging was calculated using the Karl Pearson method in Microsoft Excel, and all other measurements were performed in Microsoft Excel 2019.

3. Results and Discussion

For the first time, the effect of different pH values (2, 4, 7 and 9) of aqueous extracts on total phenol content, flavonoids, DPPH radical scavenging activity and total antioxidant capacity in the roots of Mulhatti was examined. The data obtained showed great differences. The highest amount of phenols was found in aqueous extracts of pH 7, i.e., 19.25 mg GAE/g, followed by aqueous extracts of pH 9 (17.25 mg GAE/g), in aqueous extracts of pH 2 (14.62 mg GAE/g) and in aqueous extracts of pH 4 (8.89 mg GAE/g). The effect of different pH values (3, 4, 5, 6 and 7) on phenolic content in an aqueous extract of leaves of Algerian *Matricaria pubescens* was studied, and results showed variations with pH and reported that the highest amount of phenolic compounds is present at pH 5 (9.76 ± 0.32 mg GAE/g DW) followed by pH 7 (6.94 ± 0.29 mg GAE/g DW), pH 6 (6.75 ± 0.32 mg GAE/g DW) and pH 4 (5.5 ± 0.22 mg GAE/g DW) and the lowest at pH 3 (3.81 ± 0.11) mg GAE/g DW [12]. The increase in the amount of phenolics at low pH (3-5) could be due to the inhibition of the enzymatic oxidation of phenolics or may also be due to the maintenance of the extracts [13]. The highest content of flavonoids was found in aqueous extract with a pH value of two, i.e., 5.39 mg CE/g, followed by aqueous extracts with a pH value of seven (3.02 mg CE/g) and in aqueous extracts with a pH value of nine (1.79 mg CE/g) and subsequently [14] examined the effect of the pH value in aqueous extracts with a pH value of four (1.40 mg CE/g) the extraction yield of flavonoids in Citrus medica peel extract made in various organic solvents (methanol, ethanol, ethyl acetate and water) at different pH values (3, 4, 5, 6, 7, and 8). They observed that the extraction yield of flavonoids also did not show a regular trend as present research analysis. Graphically the effect of different pH values on total phenolics, flavonoids and total antioxidant capacity in aqueous extracts of roots of Mulhatti was shown in Fig. 2.

DPPH activity to scavenge free radicals in Mulhatti roots was the highest in aqueous extracts prepared at pH 7, ranging from 11.03% to 86.83%, then at pH 2 ranging from 21.20% to 86.39%, at pH 4, ranging from 10.63% to 83.36% and pH 9 in the range from 0.10% to 85.97% and the concentration was taken in a range of 5-500 µg/mL. The corresponding IC_{50} values (µg/mL) were 82.22, 110.40, 111.99 and 146.24, respectively, and the IC_{50} value of the reference standard (ascorbic acid) was 59.52 µg/mL in distilled water (Table 2). Other researcher also studies on *Phoenix dactylifera* L.

leaf extract at different pH values for the estimation of phenols, flavonoids, DPPH radical elimination activity and total antioxidant capacity and the obtained results showed a variation of the grid in the data as in present results [15]. The opposite results were obtained by products at alkaline pH in coca [16]. The effect of pH on the antioxidant activity of *Cassia alata* L. Roxb. was studied and results obtained showed a higher antioxidant potential at pH 4 compared to other pH values (5, 6, 7, 8 and 9). They observed that as the pH increases, poor antioxidant activities are caused [17], while other researchers found that the amount of phenolic compounds and antioxidant activity increased with increasing pH [18]. Differences in the antioxidant activity of phenolic compounds may also depend on the position and number of hydroxyl and methoxy groups observed [19].

The total antioxidant capacity was the highest in aqueous extracts with a pH of two (9.93 mg AAE/g), then in aqueous extracts with a pH of four (5.54 mg AAE/g), in aqueous extracts with a pH of seven (5.34 mg AAE/g) and in aqueous extracts with pH 9 (4.23 mg AAE/g). They also determined the total antioxidant capacity in *Phoenix dactylifera* L. at different pH values of aqueous extracts and the maximum antioxidant capacity was at pH 4 (68.34 ± 0.71 mg AA/g dried extract) followed by pH 6 (64.12 ± 1.08 mg AA/g dried extract), pH 5 (63.62 ± 0.69 mg AA/g dried extract), pH 7 (62.81 ± 0.73 mg AA/g dried extract), pH 3 (55.92 ± 0.60 mg AA/g dried extract), then at pH 2 (55.11 ± 0.60 mg AA/g dried extract) [14]. These results showed large differences in total antioxidant capacity at different pH values, as in the present research data. Another reason for the different results of these phytochemicals and the antioxidant potential as well as the total antioxidant capacity can be traced back to changes in the pKa values of the reaction and the deprotonation of phenolic compounds can influence the thermodynamics of the transfer of the hydrogen [20, 21]. The lowest

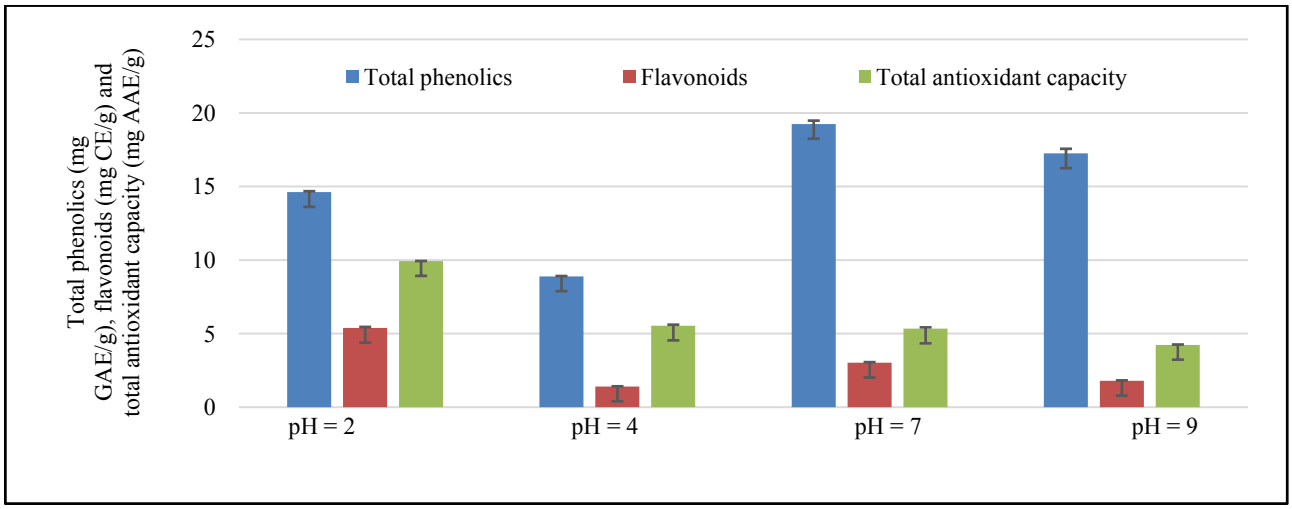

Fig. 2 Effect of different pHs on total phenolics, flavonoids and total antioxidant capacity of Mulhatti roots in aqueous extracts.

Table 2 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (%) and IC_{50} value (µg/mL) of aqueous extracts of Mulhatti roots at different pH levels.

pH	DPPH free radical scavenging activity at different concentrations (µg/mL)							IC_{50} (µg/mL)
	500	250	100	50	25	10	5	
2	86.39	81.49	45.41	33.70	23.10	21.20	a	110.40
4	83.36	83.19	43.91	28.99	17.32	12.35	10.63	111.99
7	a	86.83	54.98	36.45	23.43	13.94	11.03	82.22
9	85.97	68.33	37.50	26.67	21.11	15.42	0.10	146.24

"a" represents absence of DPPH free radical scavenging activity.

IC_{50} of DPPH free radical scavenging activity will show the highest antioxidant activity, meaning that an increase in total phenols and flavonoids causes an increase in antioxidant activity, as evidenced by a lower IC_{50} of DPPH value. On correlation between total phenols, flavonoids and DPPH free radical scavenging activity, and between total phenols, flavonoids and total antioxidant capacity of aqueous extracts of Mulhatti roots at different pH values, Pearson correlation analysis was performed. The value of the Pearson correlation coefficient (r) was given. The Pearson correlation coefficient was significantly negative when $0.61 \leq r \leq -0.97$ and significantly positive when $0.61 \leq r \leq 0.97$ [22]. Total flavonoids in Mulhatti roots had a significant and strong negative correlation with their total antioxidant capacity ($r = -0.912$, $p < 0.01$) and it can be predicted that flavonoids are the main contributor in total antioxidant capacity of Mulhatti roots by phosphomolybdenum method.

4. Conclusions

From the present study, it could be concluded that the phytochemical and DPPH free radical scavenging activity and the total antioxidant capacity were significantly affected by different pH values (2, 4, 7 and 9) and data research clearly indicated that different amounts of phenolics, flavonoids, DPPH free radical scavenging activity and total antioxidant capacity were shown by aqueous extracts of different pH levels. The maximum phenolics were present at pH 7 and pH 2 is the excellent factor for flavonoid content. In Mulhatti roots, the lowest IC_{50} value at pH 7 showed stronger scavenging activity. So pH 7 is best for antioxidant activity. The phenolic compounds present are responsible for the antioxidant activity, since the present study provides strong evidence of significant total phenolic content and radical scavenging activity significantly higher at pH 7 of aqueous extracts. At pH 2, the total antioxidant capacity was found to be higher than that of other pH values in Mulhatti roots.

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