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PHYTOCHEMICAL POTENTIAL, ANTIOXIDANT ACTIVITIES AND ENZYME INHIBITORY PROPERTIES OF CRUDE EXTRACT FROM GISEKIA PHARNACEOIDES LINN (MOLLUGINACEAE).

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Abstract:
The present work determine the phytochemical potential, antioxidant activity and enzyme inhibition activities of crude extract of Gisekia Pharnaceoides Linn (Molluginaceae).Total phenolic content (TPC) and total flavonoid content (TFC) of the plant extracts were in the range 5.56 ± 1.30--8.11 ± 1.249 mg gallic acid equivalent/g extract and 4.33 ± 1.003--6.31 ± 0.022 mg rutin equivalent/g extract, respectively. Antioxidant actions of crude extracts were evaluated by 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH). The half-maximal inhibitory concentrations (IC_{50}) of the extracts of tyrosinase and α-glucosidase were expressively higher than the references kojic acid and acarbose, respectively. These results suggest that the plant material possess moderate antioxidant activity and thought-provoking inhibitory action against key enzymes that might be due to the existence of phytoconstituents, thus supporting folkloric use.

Keywords: *Antioxidant, Phenolic, Flavonoid, Inhibition, Cholinesterase, Tyrosinase, α-Glucosidase, Gisekia Pharnaceoides.*

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INTRODUCTION:

Pakistan has been described as one of the countries that have the richest floral biodiversity worldwide. Indeed, this is due to its unique geographical location, climatic conditions, and geo morphological characteristics. Herbal medicinal systems, knowledge and practices have been communicated over the ages. For centuries, medicinal plants were the only resources available for the treatment of several diseases which plagued humanity. In fact, many of today's drugs have been derived from medicinal plants [1]. Additionally, the World Health Organization has reported that 80% of the world's population relies on herbal remedy for primary health care [2]. Conversely, to the best of our knowledge, numerous medicinal plants used as vernacular medicine have no conventional scientific consideration yet. *Gisekia pharnaceoides* Linn (Molluginaceae) is one of them, which require scientific attention. Whole plant of *G. pharnaceoides* is used as aperients, laxative , anthelmintic , in female diseases., malfunctioning semen., destroys fat and in malfunctioning of sex organs and plant extract used for killing roundworms [3].Traditionally., the plant is used on swellings. Its powder is also mixed with other herbs as poultices for sores on cattle. Extracts of the plant is used in painting and decoration of hands and the sole of feet by women due to the reddish-wine tinge color [4]. In the present investigation, dichloromethane and methanol extracts of *G. pharnaceoides* were evaluated for their conceivable antioxidant activities and inhibitory action on cholinesterase, tyrosinase and α-glucosidase.

EXPERIMENTAL:

Collection and Extraction of Plant Material

Plant material was collected in April 2015 from a farmland at Khanewal in Punjab., Pakistan. Professor Dr, Zafrulah identified the plant as *G. Pharnaceoides* and a specimen voucher (No Stewart 234) was deposited at Institute of Pure and Applied Biology., Bahauddin Zakariya University., Multan., Pakistan. For the purpose of effective extraction, whole plant material was shade dried for 15 days. Then dried plant material was ground in blender and weighed. The extraction of this finely ground material was affected by simple maceration. The maceration of powdered whole plant (800g) was done in dichloromethane (1.5 L) for a period of 24 h separately and then filtered. The procedure was repeated thrice using 1.5 L dichloromethane in each step. The filtrates from each step were combined and concentrated using rotavapor (Buschi, Switzerland) at 35 °C. Same procedure was adopted for the preparation of methanol extract. Yield of crude dichloromethane extract is 18.3 g., while the yield of methanol extract was 50.5 g.

Chemicals

DPPH (1,1-diphenyl-2-picryl hydrazyl) radical and rutin were purchased from Sigma Aldrich Chemical Company, USA; Folin and Ciocalteau's Phenol reagent and Trichloroacetic acid (TCA) from Qualikems Fine Chemical Pvt. Ltd. Gallic acid monohydrate from Kem Light Laboratories Pvt. Ltd., Mumbai, India. Solvents and other chemicals used for this study were of analytical grade, while water was glass distilled.

Preliminary phytochemical screening of plant material

The withered and pulverized plant material was explored for the identification of alkaloids, glycosides, flavonoids and terpenoids in plant material. The tests engaged are given below.

Test for alkaloids

Brain and Turner [5] described the finding of alkaloids. Three gram of the minced plant material was simmered with ten ml of acidified water in test tube for 1 min, cooled and permitted the debris to settle. Screen the fluid in a test tube. One ml of this filtrate was taken and three drops of dragendorff's reagent was added, there was no precipitate. The residue of filtrate was made alkaline by adding dilute ammonia solution. It was shifted to separating funnel and 5 ml of chloroform solution was added, two layers were observed. The inferior chloroform layer was pipetted out into another test tube. Chloroform layer was extracted with 10 ml of acetic acid and then discarded the chloroform. Extracts was divided into three portions; to one portion added few drops of dragendorff's reagent and to second few drops of Mayer's reagent was added. Turbidity or precipitate was related with the third untreated control portion.

Test for anthraquinone glycosides

One gram of minced plant material was taken and extracted with ten ml of hot water for five minutes, permitted it to cool and filtered. Scum was extracted with ten ml of carbon tetrachloride. Then carbon tetrachloride layer was taken off, washed it with five ml water and then five ml insipid ammonia solution was added. No free anthraquinones was revealed as absence of appearance of pink to cherry red color in the ammonical layer. One gram of subsequent sample of the same plant material was extracted with ten ml of ferric chloride solution and 5 ml of hydrochloric acid then it was heated on water bath for ten minutes and filtered. Filtrate was cooled and treated as above. [5].

Test for cardioactive glycosides
One gram of milled plant material was taken in a test tube and ten mL of 70% alcohol was added. It was then boiled for two minutes and filtered. Filtrate was diluted twice of its volume with water and then one ml of robust lead subaceatate solution was added. This treatment leads to the precipitation of chlorophyll and other pigments, which was then filtered off. Filtrate was extracted with an equal volume of chloroform. Chloroform layer was pipetted out and evaporated to dryness in a dish over a water bath. Residue was dissolved in 3 mL of 3.5% ferric chloride in glacial acetic acid and was transmitted to test tube after leaving for 1 min. 1.5 ml of sulphuric acid was then added, which formed a distinct layer at the bottom. Cardio active glycosides was revealed the appearance of brown color at boundary (due to deoxy sugar) on standing, and appearance of pale green color in the upper layer (due to the steroidal nucleus) [5].

Test for flavonoids
Two g of the air dried milled plant material was boiled with twenty ml of distilled water for ten minutes and filtered. The filtrate was acidified with few drops of dilute HCl. Took 5 ml of aliquot of the filtrate and made it alkaline (pH 10) with sodium hydroxide (T.S), A yellow colour was established showed the probable occurrence of flavonoids [7].

Test for terpenoids
Plant material was thawed in two ml of chloroform and evaporated to dryness. To this, two ml of rigorous sulphuric acid was added and heated for about 2 minutes. A grayish colour signposted the presence of terpenoids [6].

Total phenolic content (TPC) [8-9]
Spectrophotometric procedures are most generally used for the quantification of phenolic content. Assessment of total phenol content in the plant extract was measured spectrophotometricly by Folin–Ciocalteu colorimetric method, using Gallic acid as the standard and articulating results as gallic acid equivalent (GAE) per gram of sample. Diverse concentrations (0.01-0.1 mg/ml) of gallic acid were prepared in methanol. Aliquots of 0.5 ml of the test sample and each sample of the standard solution were taken, mixed with two ml of Folin–Ciocalteu reagent and 4 ml of inundated solution of sodium carbonate (7.5% w/v). The tubes were covered with silver foils and incubate at room temperature for thirty minutes with intermittent shaking. The absorbance was taken at 765 nm using methanol as blank. All the samples were scrutinized in three repetitions. The total phenol was determined with the help of standard cure prepared from pure phenolic standard (gallic acid). Folin–Ciocalteu is a very sensitive reagent containing phosphomolybdate and phosphotungstate that form blue-complex in alkaline solution by the bargain of phenols. This blue color was measured spectrophotometricly.

Total flavonoid content (TFC) [10]
The TFC of the plant extract was indomitable by aluminum chloride colorimetric assay. Briefly, 0.5 ml aliquots of the extract and standard solution (0.01-1.0 mg/ml) of rutin were added with two ml of distilled water and subsequently with 0.15 ml of sodium nitrite (5% $NaNO_2$, w/v) solution and mixed. After 6 minutes, 0.15 ml of (10% $AlCl_3$, w/v) solution was added. The solutions were allowed to stand for further 6 min and after that 2 ml of sodium hydroxide (4% NaOH w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with immediate addition of distilled water, mixed systematically and permitted to stand for another 15 min. The absorbance of each mixture was resoluted at 510 nm against the same mixture but without seed extract as a blank. TFC was determined as mg rutin equivalent per gram of sample with the help of calibration curve of rutin. All determinations were performed in triplicate (n=3).

Determination of Antioxidant Activity

DPPH Free Radical Scavenging Test
The inference of the plant extracts on DPPH radical was evaluated according to the method described by Sarikurkcu [11]. Briefly, one mL of plant extract was added to 4 mL DPPH solution (0.004%) in methanol. The absorbance was measured at 518 nm after thirty min incubation at room temperature in the dark. Percentage DPPH scavenging activities of the extracts and reference standards were determined using the formula
% scavenging activity $100 - [(A_s - A_b) / A_c \times 100]$

Where A_s = Absorbance of sample (extract or reference standard), A_b = Absorbance of blank and A_c = Absorbance of negative control

Determination of Cholinesterase, Tyrosinase and α-Glucosidase Activity
Cholinesterase Inhibition Assay
Cholinesterase inhibitory activity was measured using Ellman's method as formerly reported by Aktumsek et al (12) with slight modifications. The plant extract (50 µL) was mixed with dithiobisnitro-benzoate (DTNB) (125 µL) and cholinesterase solution (25 µL) in Tris-HCl buffer (pH 8.0) in a 96-well micro plate. The reaction was originated by the addition of 25 µL of acetylthiocholine iodide or

butyrylthiocholine chloride. The absorbance was read at 405 nm after 10 min incubation at room temperature. Galantamine is used as a positive control and IC_{50} value was determined.

Tyrosinase Inhibition Assay

Tyrosinase inhibitory activity was measured using the modified dopachrome method earlier described by Orhan et al [13] with slight modifications. Plant extract (25 µL) was mixed with tyrosinase solution (40 µL) and phosphate buffer (pH 6.8) (100 µL) in a 96-well micro plate and incubated for 15 min at 37°C. L-DOPA (40 µL) was then added to the mixture to initiate the reaction. The absorbance was read at 492 nm after 10 min incubation at 37°C. Kojic acid is used as a positive control and IC_{50} was calculated.

α-Glucosidase Inhibition Test

α-Glucosidase inhibitory activity was performed following the previous method described by Palanisamy et al [14] with some modifications. Plant extract (50 µL) was mixed with glutathione (50 µL)., α-glucosidase solution (50 µL) in phosphate buffer (pH 6.8)., and PNPG (50 µL) in a 96-well micro plate and incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.2 M sodium carbonate (50 µL) and the absorbance was read at 400 nm. Acarbose is used as a positive control and IC_{50} was determined.

Statistical Analysis

The experiments were carried out in triplicate. The results are expressed as mean ± standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with using SPSS v. 14.0.

RESULTS:

Preliminary phytochemical screening

Phytochemical test were performed for finding secondary metabolites i.e. alkaloids, anthraquinone glycosides, cardiac glycosides, flavonoids and terpenoids in plant material. The results of the study are shown in table1.

Quantification of Phenolic content

The total phenol and flavonoid content of the plant extracts are summarized in table 2. Dichloromethane extract of *G. pharnaceoides* (PWGPD) has higher phenolic content than methanol extract of *G. pharnaceoides* (PWGPM). On the other hand, it was observed that the flavonoid content of the methanol extract of *G. pharnaceoides* (PWGPM) showed the highest flavonoid content shown in table 2.

Table 1: Results of phytochemical screening of *Gisekia Pharnaceoides*

Plant name	Part used	Alkaloids	Anthraquinone glycosides	Cardiac glycosides	Flavonoids	Terpenoids
Gisekia Pharnaceoides	Whole plant	+	-	-	++	+

+: Normal presence. ++ Maximum presence. - : Absence.

Table 2: Total phenol and flavonoid content of the plant extracts.

Plant extracts	Total phenol content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
WPGPD	5.56 ± 1.30	4.33 ± 1.003
WPGPM	8.11 ± 1.249	6.31 ± 0.022

WPGPD: whole plant *G. Pharnaceoides* dichloromethane extract; WPGPM: whole plant *G. Pharnaceoides* methanol extract.

Table 3: Antioxidant activity of extracts

Plant extracts/ control	IC50 ± SEM. µg/ml
WPGPD	4.38 ± 1.431
WPGPM	0.28 ± 0.004
Trolox	0.30 ± 0.002

WPGPD: whole plant *G. Pharnaceoides* dichloromethane extract; WPGPM: whole plant *G. Pharnaceoides* methanol extract.

Antioxidant Activities

Table 3 recapitulates the radical scavenging of *G. pharnaceoides*. It was found that the plant extracts showed variable radical scavenging capabilities on DPPH. Methanol extracts of *G. pharnaceoides* (IC_{50}: 0.28., mg/mL) significantly ($p<0.05$) scavenged as compared to the positive control trolox (IC_{50}: 0.30 mg/mL). On the other hand, it was observed that the dichloromethane extracts of *G. pharnaceoides* (IC_{50}: 4.38., mg/mL) scavenged but were significantly ($p<0.05$) less active than trolox (IC_{50}: 0.30 mg/mL) results shown in table 3.

***In-vitro* enzyme inhibition**

It was observed that the plant extracts exhibited variable inhibitory effects on cholinesterases, acetyl cholinesterase and butyryl cholinesterase (Table 4). The plant extracts were significantly ($p<0.05$) less active than the positive control eserine against cholinesterases. However, both dichloromethane and methanol extracts of *G. pharnaceoides* significantly ($p<0.05$) inhibited tyrosinase (IC_{50}: 2.63 mg/mL; IC_{50}: 2.50 mg/mL resp) and α-glucosidase (IC_{50}: 4.67 mg/mL; IC_{50}: 2.53 mg/mL resp) as compared to kojic acid (IC_{50}: 4.04 mg/mL) and acarbose (IC_{50}: 5.87 mg/mL) respectively.

Table 4: Inhibition concentration of the plant extracts on cholinesterases., tyrosinase and α-glucosidase.

Plant extracts/positive controls	IC_{50} (mg/mL)			
	Cholinesterases			
	Acetylcholinesterase	Butyrylcholinestere	Tyrosinase	α-Glucosidase
WPGPD	1.44 ± 0.001	3.29 ± 0.018	2.63 ±0.035	4.67 ± 0.016
WPGPM	1.50 ± 0.044	NA	2.50 ±0.014	2.53 ± 0.051
Eserine	0.04± 0.000	0.02 ± 0.001	ND	ND
Kojic acid	ND	ND	4.04 ±0.001	ND
Acarbose	ND	ND	ND	5.87 ± 0.200

WPGPD: whole plant *G. Pharnaceoides* dichloromethane extract;WPGPM: whole plant *G. Pharnaceoides* methanol extract. Values significantly ($p < 0.05$) lower than the positive control. Values significantly ($p < 0.05$) higher than the positive control; NA: not active; ND: not determined

DISCUSSION:

Plant bioactive compounds have played a vital role universally in avoiding and curing several human illnesses. It is because of their broad spectrum of chemical and biological activities. All medicinal plants require a thorough investigation before their manipulation as medicine because the therapeutic prospective entirely depends on the quality of plant material used and the study of any crude sample material of natural origin is advantageous only if it contains the active constituents which have to be recognized to validate its real value [15]. Moreover, information about different phyto-constituents of plants is a very important and advantageous as it is much valuable in the production of complex chemical compounds as well as screening of their biological activities. The present study has been carried out for the phytochemical potential, quantification of the total phenolic, flavonoid, of *G Pharnaceoides* Linn.

The use of plant-based products for the management and treatment of diseases is gaining much impetus from both scientific and consumer perspectives. Indeed, herbal therapies have been used for curative purposes since the dawn of civilization. The relentless efforts for wellbeing and to combat diseases have guided scientists as well as health care providers towards safer and natural alternatives such as medicinal plants. Currently., there is a transformed interest in natural inhibitors from plant-based medicines to modulate physiological effects of enzymes linked to several pathologies such as diabetes., obesity., neurodegenerative diseases., and inflammation., amongst others. The present study has endeavored to investigate the possible inhibitory effects of G. pharnaceoides to modulate key enzyme involved in diabetes (α-glucosidase)., neurodegenerative disorders (tyrosinase, acetylcholinesterase, and butyrylcholinesterase), and melanogenesis (tyrosinase). Plant extracts from the current study were found to inhibit acetylcholinesterase although their inhibitory action was less potent than the known inhibitor eserine. The variation in activity of the plant extracts against these enzymes might be explained based on the complex composition and potential synergistic effect(s) of individual phytochemicals present in each sample. Interestingly, we found varying concentration of phenolics and flavonoids in extracts of the plant. Previously., it has been reported that inhibitory activity of plant extracts might be due to the presence of several phytochemicals such as flavonoids., saponins., and tannins. Additionally, studies on α-glucosidase inhibitors isolated from medicinal plants suggest that several potential inhibitors belong to flavonoid class which has features of inhibiting metabolic enzymes. Recently, it has been shown that phenolics play a role in mediating amylase inhibition and therefore have potential to contribute to the management of type 2 diabetes [16].

Free radicals are known to play a pivotal role in the onset and exacerbation of several pathologies . By counteracting these free radicals., antioxidants help in preserving good health. Indeed., phytochemicals have received much interest owing to their molecular structure which consists of hydroxyl groups on aromatic rings and this has been associated with their functionality as oxidant scavengers [17]. Phytochemicals act by inhibiting oxidative chain reactions at cellular level thereby increasing their therapeutic efficacy [18]. In the present study, the phenolic content of the plant extracts was estimated using the Folin-Ciocalteau method. This method is rapid and simple but also measures various interfering nonphenolic compounds such as ascorbic acid, thiol, and nitrogen containing compounds [19]. Flavonoids are the major class of phenolic compounds and are known to exhibit strong antioxidant activities [20-21]. Interestingly, in the present study, it was observed that Oh extract showed high phenolic and flavonoid content. The reducing power of plant extracts is regarded as an indication of their antioxidant capacities [22].The free radical quenching potential of the plant extracts was determined using two nitrogen-centered radicals. Results from the present study have demonstrated that the plant extracts showed good abilities to quench DPPH. The ability of the plant extracts to quench and was related to the observed high phenol content.

CONCLUSION:

Data gathered from the present investigation demonstrated that methanol and dichloromethane extract possessed antioxidant capabilities and exhibited inhibitory potential against cholinesterase, tyrosinase and α-glucosidase in vitro. Furthermore, to date, no such scientific information on this plant has been gathered. However, it was observed that the antioxidant capacities and cholinesterase inhibitory activities of the plant extracts were less potent than the controls. Further works related to the isolation of the active constituents through bioassay-directed fractionation are in progress in our laboratory. To mark those compounds that can be exploited for novel drug development and to be used as inhibitors of enzymes of therapeutic importance.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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