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Preliminary Phytochemical screening and Pharmacological assessment of *Medicago italica* Mill

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The current research work was performed to screen the existence phytochemicals and to evaluate pharmacological activities of *Medicago italica* to prove its utilization in the conventional medicine for treatment of many diseases in sub-continent. In this study *M. italica* methanolic crude extract was screened for antioxidant, antidiabetic, antibacterial and antifungal bioassays. For antioxidant activities, DPPH stable free radicals, ABTS radical cation, H_2O_2 and Na_2MoO_4 tests were used. The extract possessed antioxidant activity. Highest antioxidant potentiality was found against ABTS with IC_{50} 98µg/mL, followed by MoO_4 100µg/mL, DPPH 149µg/mL and H_2O_2 250µg/mL. The average phenolic and flavonoid contents of the plant were 5.48mg GA/g and 6.88mg RU/g respectively. The extract at 500µg/ml inhibited growth of *Escherichia coli, Klebsiella pneumoniae* and *Bacillus subtilis,* at the rate of 12±1.80, 11±1.09 and 14±0.91 mm respectively. Likewise, growth of fungal strains like *Aspergillus niger, Aspergillus fumigatus* and *Aspergillus flavus* was inhibited to 57, 70 and 43% at concentration of 500µg/ml. respectively. This study scientifically validate the potential of *M. italica* as a good source of saponins, polyphenolic, alkaloids and tannins

Keywords: *Medicago italica,* antioxidant, phytotoxic, antibacterial, antifungal

INTRODUCTION

Medicinal plants have a very key role in keeping the standard of human health. They play very essential role in controlling different chronic diseases because of the presence of different bioactive compounds (Kooti et al. 2014a). The plants derived medicines have a key role in both traditional and modern healthcare. It can be noted from the fact that medicinal plant measures are used from hundreds of years (Reshma, 2021). As the underdeveloped countries have no access to use modern medicine and therefore, large proportions of the population in under developed countries rely on the traditional practices of medically important plants for executing medical-care needs (Vishwakarma et al. 2013; Manzano and Agugliaro, 2020). Even though, modern and traditional medicines run side-by-side. Herbal medications have regularly maintained their magnetism for cultural and historical reasons (Vishwakarma et al. 2013). Even, in East and Central Africa people uses medicinal plants and traditional medicine practices to overcome healthcare problems (Bussmann et al. 2018; Kigen et al. 2019).

Medicinal herbs are a good alternative to chemical drugs. The main reasons for this are low side effects than chemical drugs and also their ingredients cause biological balance and avoid drug accumulation in the body (Asadi-Samani et al. 2013; Kooti et al. 2014b). Also, the plant antioxidant properties reduce drugs toxicity (Kooti

et al. 2014c; Asadi-Samani et al. 2015). The World Health Organization (WHO) also recommend herbal medicines as an available, inexpensive and culturally adequate form of health-care consumed by huge number of people, that stand out to manage with the tenacious chronic non-transmissible diseases in the middle of rising medical-care expenses and virtually universal severity (WHO, 2020). At present, medicinal herbs and their extracts are extensively used to reduce diverse human diseases.

Since 19th century, various groups of secondary metabolites cyanogenic glycoside, phytic acid flavonoids, phytoestrogens, gossypol, and carotenoids have been identified and formulated. These metabolites are used in the production of modern medicine or served for new drugs discovery (Uddin et al. 2011). The world has viewed increasing scientific and marketable benefit in medicinal plants and their products (Srinivasan et al. 2006; Ali and Jahangir, 2001). In reality, it could be stated that plants having medicinal properties may have co-evolved with humans. Management of numerous diseases, such as diabetes and cancer etc, is now dependent upon natural products (Hung et al. 2012; Basmadjian et al. 2014).

Medicago belongs to family Fabaceae. Commonly called as medick or burclover (Steele et al. 2010; Gholami et al. 2014). *Medicago italica* plant is prostrating annual and hairy. The branches long ranges between 10–35 mm. Leaves are 3-foliolate, leaflets are obovate and the terminal leaflets are 10–17 mm long and 9–16 mm in width. Inflorescences contains 4–10-flowers, quite rare 15-flowered; peduncle half as long as subtending petiole. Calyx teeth are longer than tube. Corolla is yellow, standard 5–10 mm long; keel longer than wings. Pod is cylindrical to truncate-conical or discoid and is about 2–7 mm long and 4–10 mm in diameter. Coils are 3–8 and the seeds are 4–9.

The plant is used for the treatment of epilepsy, leucorrhoea, rheumatism sciatica and contraceptive. Their leaves were used to treat blood dysentery, while the root and seeds were consumed for removal of dysentery, germicide and rheumatism. Traditionally the roots were consumed for malaria, fever, piles and to remove the earlier cough (Moon., 2010). On the behalf of these highlights the current study attempts to examine antioxidant, antifungal and antibacterial activities of *M. italica* methanolic extract.

MATERIALS AND METHODS

Identification and Extraction

Mature plants were taken from the local area of Bannu, Pakistan during February. The taxonomic detection of the plant was endorsed by Prof. Abdur Rehman, the Taxonomist. The plant was submitted in the herbarium of Botany department, University of Science and Technology. Bannu under voucher number Bot. 01(BUH).

The plants were perfectly washed with distilled water and dried under shade at room temperature for about 12 days and powdered via a grinder. Two hundred gram powder was extracted using methanol of analytical grade. A plant cellulosic cell wall must be removed in order to emancipate the internal cell content, so slight shaking was provided for 12 hours. The extract was filtered and processed through rotary evaporator to take out all organic solvents. The leftover residue was stored for further experimentation.

Stock and Running Solutions

Crude extract (5mg) dissolved in 5mL methanol served a stock solution, which was further diluted into 100, 250, 500 and 1000µg/ml by adding methanol ($M_1V_1= M_2V_2$).

Phytochemical Testing

Trease and Evans, 1989 (modified) method was used to test for the presence of phytochemicals. Positive detection of flavonoids, alkaloids, tannins and saponins were observed as presented in Table 1.

Estimation of Total Phenolic Content in Crude Extract

The phenolic compounds of the methanolic extract were analyzed using Folin–Ciocalteu method (Everette et al. 2010). About 200 µL of crude extract (1 mg/mL) were taken in 3 mL with distilled water. About 0.5 mL of Folin–Ciocalteu reagent were mixed and left for 3 min, than 2 mL of 20% (w/v) sodium carbonate was added. The mixture was allowed to stand for one hour in dark, and absorbance was measured at 765 nm. As shown in Fig 1, the calibration curve gave the total phenolic and the results were recorded as mg of gallic acid equivalent per g dry weight.

Determination of total flavonoid contents in plant extract

Total flavonoids contents (TFC) were analyzed according to the standard procedure of (Sakanaka et al. 2005). Briefly, 250 µl of crude extract (1-5 in methanol) and rutin (1-5 in methanol) was mixed with 1250 µl dH_2O. Than $NaNO_2$ solution of 75 µl of 5% (w/v) was added. At incubation for 6 min, 150 µl of 10% (w/v) $AlCl_3.6H_2O$ was added and kept for 5 min. Finally 500 µl of 1M NaOH was added and the mixture was added up to 2500 µl with dH_2O and shaken vigorously. Absorbance was recorded at 510 nm. The results were expressed as rutin equivalent mg/g of the dried extract (Fig 2).

Antibacterial Activity (agar diffusion method)

Antimicrobial activities of raw extract was find out according to the protocol of (Duraipandiyan and Ignacimuthu, 2009). Concisely three bacterial strains, two Gram-negative *Escherichia coli, Klebsiella pneumoniae* and one Gram-positive *Bacillus subtilis* were obtained from Khalifa Gul Nawaz Hospital, Bannu. Suspensions of the bacteria were set by caring-1 CFU of bacterial pathotype from the preserved slants in 0.9% NaCl solution. The suspensions were incubated at 37ºC for 24 hours. Nutrient agar of 6.5g was balanced and liquified in distilled water (100mL). The sample was autoclaved for 20 minutes at 121ºC and then cooled at room temperature. To each petri plate 30mL media were transferred and kept to solidify. From each bacterial suspension decent volume was taken by swab and spread slightly on the petri plates. Five wells were dug per plate with a sterile cork borer (3-6 mm), 1 central hole surrounded by 4 ones. All the wells were appropriately categorized. DMSO (67µL) was added in the central hole and plant extract (final concentration of 200µg/mL and 500µg/mL) as well as cipval (final concentration of 10µg/mL in DMSO) in the surrounding holes. DMSO served as negative control. Inhibition zones were calculated after a day with a graduated ruler. A clear zone of bacterial inhibition was observed around all holes. The diameter of the clear zone was measured in mm. Inhibition by active ingredients of the extract was determined by measuring linear growth (mm) in tests with reference to cipval (mm), a standard antibiotic (positive control).

% Inhibition= Inhibition in test/ Inhibition control*100

Antifungal bio-assay

Sabourad Dextrose Agar (SDA) media was autoclaved at 121ºC and cooled to 40-50ºC. 7 ml of SDA along with the extract was added to each test tube, i.e. 67µL to every test tube (final volume of 200 and 500µg/ml). Then the solution was left for drying. The negative control test tubes were applied with DMSO only while the positive control with antifungal drug terbinafine. The tubes were then incubated at room temperature inside the laminar flow cabinet for solidification and the fungal inocula were freckled uniformly. The overall set mixture was placed at 30ºC in an incubator with open water in a tub. The inhibited fungal growth was detected after 7 days.

% Inhibition= Inhibition in test/ Inhibition control*100

Antioxidant assay

DPPH activity

The DDPH (1, 1-diphenyl-2-pierythydrazyl) activity was done using the method as suggested by Gyamfi et al. 1999 a little modifications. DPPH absorbance was calculated using spectrophotometer at 517nm which was 0.708 (<1). This reaction was carried out in darkness at 37°C. From each sample, 200µL extract was taken and mixed with 800µL DPPH was mixed. The sample was allowed to react for 30 minutes in darkness at 25°C and its absorbance was checked. For ascorbic acid, the above procedure was repeated. The amount of free radicals was expressed as IC_{50}. Ascorbic acid was used as control. The procedure was done thrice. The activity was planned on the percent amount of scavenged DPPH radicals:

$$\%\ DPPH\ scavenging = 1 - \frac{Abs\ of\ DPPH - Abs\ of\ sample}{\text{DPPH Abs}} \times 100$$

Abs of DPPH= absorbance of DPPH, Abs of sample= absorbance of the extract.

ABTS$^+$ radical cation (Potassium per sulfate) bioassay:

Potassium per sulfate bioassay was performed for ABTS$^+$ radical cation according to the procedure of (Arnao et al. 2001). The main objective of the ABTS bioassay was the ability of extract to search 2, 2′- azino-bis (ethylbenzthiazoline-6-sulfonic acid) radical cation. An amount of 7mM ABTS and 2.45mM

potassium per sulfate were mixed and kept to react for 30 minutes. Later, 1.8mL $ABTS^{+}$ potassium per sulfate was taken and about 0.2mL extract was added. Absorbance ($ABTS^{\cdot+}$ Potassium per sulfate) were recorded at 745nm. Ascorbic acid was used as control. Calculation of the extract was taken at different concentrations;

$$\%\ antioxidant\ capacity = 1 - \frac{Abs\ of\ test\ sample}{\text{Abs of ascorbic acid}} \times 100$$

H_2O_2 scavenging activity:

The method of Dehpour et al. 2009 (modified) was used to determine the activity of H_2O_2. About 40mM H_2O_2 solution in phosphate buffer (pH 7.4) was taken and measured at 230nm to determine its absorption. 200µL extract from each concentration (50µg/ml-500µg/ml) were added to 1.8mL of H_2O_2 solution. The solution was incubated at room temperature for 10 minutes and the absorbance was measured at 230nm against a blank, with phosphate buffer only. H_2O_2 radical was determined as;

$$\%\ antioxidant\ capacity = 1 - \frac{Abs\ of\ test\ sample}{\text{Abs of ascorbic acid}} \times 100$$

Sodium molybdate activity:

Sodium molybdate antioxidant ability was performed using the method of Prieto et al. 1999. Concisely, 3mL solution of 0.6M sulphuric acid, 28mM sodium phosphate and 4mM sodium molybdate was mixed with several concentrations of the extract. Incubation of the mixture was done at 95^0C for 90 minutes. The blend was let cooled and the absorbance was taken at 765nm using a digital spectrophotometer against a methanol blank. Ascorbic acid was used as standard. The extract Reduction power was calculated to the number of equivalents of ascorbic acid. The antioxidant capacity of *M. italica* extract was calculated by;

$$\%\ Sodium\ molydate\ reducation = \frac{Abs\ of\ test\ sample}{\text{Abs of ascorbic acid}} \times 100$$

Anti-Diabetic Assay

A modified protocol of Malik, 1980 was used for the detection of antidiabetic potential of the extract.

α-amylase activity

About 0.1g potato starch was mixed to sodium acetate buffer of 100 mL to gain starch solution of 0.1 % w/v. To prepare enzyme solution, α-amylase (27.5) mg was dissolved in 100mL of dH_2O. Colorimetric reagent was obtained by mixing 96mM of 3, 5 di-nitro salicylic acid solutions with sodium potassium tartarate. Than starch solution was added to the plant extract, mixed α-amylase and placed for a while. The temperature was kept at 25 ºC. After 3 to 4 minutes, measurement was taken. The generated maltoses were calculated by production of 3-amino-5- nitro salicylic acid that formed from the reduction of 3, 5 dinitro salicylic acid. The absorbance was recorded at 540nm using spectrophotometer.

α-glucosidase activity

The activity of α-glucosidase was done using 2% w/v starch solution as substrate. Tris buffer (0.2 M, pH 8.0) was prepared. All the samples, 200µg/ml-800µg/ml of plant extracts, starch solution and Tris buffer were incubated at 37°C for 5 min. α-glucosidase 1mL of 1U/mL was then added and kept for 40 min at 35°C. Later, addition of 2 ml of 6N HCl was used to stop reaction. Using spectrophotometer, the measurement of the color intensity was taken at 540 nm.

Calculation of IC_{50}

The *M. italica* extract required for fifty percent of the inhibition enzymes activity (IC_{50}) was determined at five different concentrations of the extract using % inhibition. The calculation was done at;

$$\%\ Inhibition = \frac{Ac - As}{\text{Ac}} \times 100$$

Ac: Absorbance of control As: Absorbance of sample.

RESULTS AND DISCUSSION

Phytochemical Analysis

Table 1 indicated the presence of tannins, flavonoids, alkaloids, saponins and phenols. Overall, phenols were shown as µg/mg gallic acid equivalent using the standard curve equation: Y = 0.1255X+0.232, R^2=0.9715, where Y: Absorbance at 765nm X: Total phenol in the crude (Figure 1). The total flavonoid content of the plant was 5550µg RU/mg, calculated using the standard curve equation: Y = 0.1331X+0.06, R^2=0.9715 (Figure 2). The occurrence of various plant secondary metabolites as alkaloids, steroids, tannins, flavonoids and phenolics etc have been detected in different plant extracts used for its phytochemicals analysis (Tsakala et al. 2006, Um

et al. 2017, Bashir and Kumar, 2020, Benali et al. 2021).

Table 1: Phytochemical analysis of crude methanolic extract of *M. italica*

Phytochemical	Result
Tannins	Present
Flavonoids	Present
Alkaloids	Present
Saponins	Present
Phenols	Present

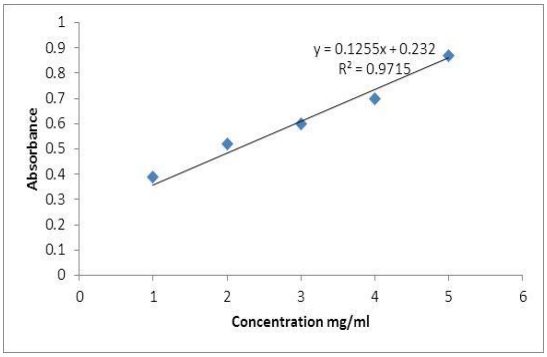


Figure 1: Standard Calibration Curve of Gallic Acid

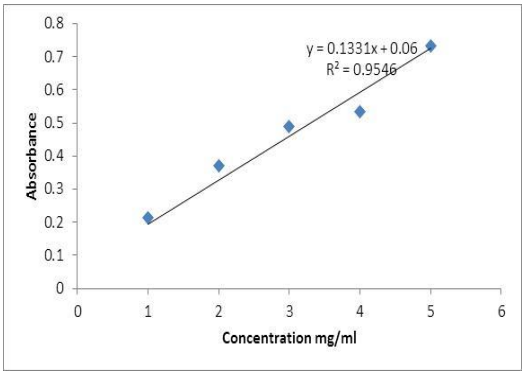


Figure 2: Standard Calibration Curve of Rutin

Antibacterial Property

Plant secondary metabolites are used to improve and develop medicines that are less toxic and more effective in exterminating pathogens. These composites have a significant function to treat human pathogens. Apart from the challenging bacterial resistance, the major problems with the present antimicrobial drugs are their high prices. Another factor is that they are not readily available at the accurate place and accurate time.

Antibacterial activities were shown at different doses of *M. italica* methanolic crude extract. At 200µg/mL the crude extract inhibited 06mm growth of *E.coli,* 07mm *K. pneumonia* and 11mm *B. subtilis*. When the extract concentration was boosted to 500µg/ml, linear growth inhibition was also observed. This was 12mm for *E.coli*, 11mm *K. pneumoniae* and 14mm *B. subtilis* (Table 2). The analysis of results clearly indicates that the plant extract was active in all cases. *B. subtilis* was most susceptible among the three. DMSO served a negative control and ciprofloxacin (10µg/mL) as a positive control.

The antibacterial activity shown by a number of plant extracts against drug-resistant *E. coli* and *K. pneumoniae* has been reported by many researchers (Tsakala et al. 2006, Um et al. 2017, Bashir and Kumar, 2020, Benali et al. 2021).

To address all these problems, related to antibacterial drugs, the researchers are seeking for novel, efficient, safe and less harmful natural remedy. To answer this problem, the current work is carried out to assess the antibacterial properties of *M. italica* with an anticipation of find an effective, harmless and an economical antibacterial medicine.

Antifungal screening

A. niger infects humans the third most often. After infection a fungal ball is produced in lungs of individuals. Similarly *A. flavus* produces mycotoxin and aflatoxin, which contaminates food stuffs. *A. fumigatus* causes disease in humans more than any other mold. To cope with these problems, the present study was conducted to explore antifungal activities of *M. italica*.

It was found that *M. italica* crude extract significantly inhibited the growth of tested fungal strains. It is therefore, considered a good and valuable source against the tested fungi. The crude extract at 200µg/ml exhibited 27% growth inhibition of *A. niger*, 50% *A. fumigatus* and 20% *A. flavus*. Consequently, higher growth inhibition was observed when concentration increased to 500µg/ml (Table 3). DMSO served a negative control. No fungal growth was observed in positive control test tubes treated with turbinafine.

Studies suggested that the plant extract has an inhibitory capacity towards microorganisms due to the presence of saponins, polyphenolic compounds like tannins and catechin (Sahreen et al. 2011, Bashir and Kumar, 2020, Benali et al. 2021).

Table 2: Antibacterial activity of *M. italica* crude extract

Tested Bacteria	Concentration (µg/ml)		
	200µg/ml	500µg/ml	10µg/ml ciprofloxacin
	Zone of inhibition in mm ± (SD)		
B. subtilis	11±1.02	14±0.91	22±0.67
E. coli	06±1.28	12±1.80	18±0.89
K.pneumoniae	07±0.87	11±1.09	17±1.00

Table 3: Antifungal activities of *M. italica* crude extract.

Fungal Strains	DMSO		Terbinafine (10µg/ml)		*M. italica* extract 200µg/ml		500µg/ml	
	Growth		Inhibition					
	cm	%	cm	%	cm	%	cm	%
A. niger	11±1.0	100	09±0.67	82	03±4.73	26	6±0.84	57
A. fumigatus	10±1.54	100	10±3.02	100	05±3.02	50	7±2.11	70
A.flavus	10±2.52	100	07±0.36	70	02±1.92	20	4±1.45	43

mm: millimeter, DMSO: Dimethyl sulphoxide
Results were expressed as Mean±SD, and in %.

Table 4 : (a) Inhibition of α-amylase activity by *M. italica*.

Sample	Concentrations (µg/ml)	α-amylase inhibition (%)	IC_{50}
M. italica	200	48±0.45	220µg/ml
	400	60±1.34	
	600	70±0.39	
	800	79±0.87	
	1000	88±1.09	
Glimepiride	2	77±0.74	1.3µg/ml
	4	83±1.01	
	6	91±0.87	
	8	95±0.86	
	10	98±0.70	

(b) Inhibition of α-glucosidase by *M. italica* crude.

Sample	Concentrations (µg/ml)	α -glucosidase inhibition (%)	IC_{50}
M. italica	200	40±0.92	240µg/ml
	400	62±1.83	
	600	74±2.80	
	800	82±2.67	
	1000	87±1.73	
Glimepiride	2	60±0.74	1.7µg/ml
	4	74±2.91	
	6	84±0.97	
	8	91±1.86	
	10	96±1.50	

Antioxidant Property

DPPH

DPPH activity of extract of *M. italica* (50-500µg/ml) was determined in all cases. Highest scavenging activity of the extract was found at 500µg/ml (70%). The calculated IC_{50} was 147µg/ml**.** The antioxidant results against DPPH

free radicals are listed in Figure 3a.

ABTS

Scavenging capacities for ABTS (free radical) of the tested samples were measured by spectrophotometer. Maximum scavenging of the extract was noted at 500µg/ml (85%), while control exhibited 92% antioxidant property at the same concentration. The IC_{50} was 80µg/mL (Figure 3b).

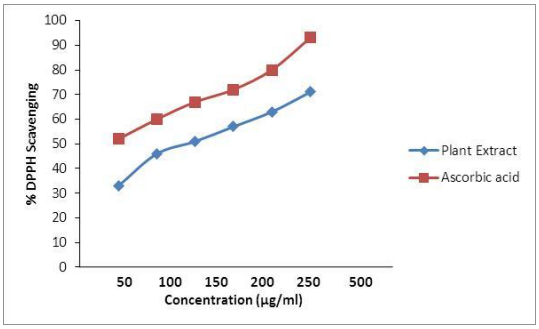
H_2O_2 reducing activity

H_2O_2 radical activity of *M. italica* methanolic extract was confirmed at different concentrations (50-500µg/ml). At 500µg/mL, 61% activity of the extract showed a significant H_2O_2 scavenging activity. The activity tends to decline with decrease concentration of the extract. Highest antioxidant activity (90%) was noted for control at 500µg/ml. The calculated IC_{50} was 215µg/mL (Figure 3c).

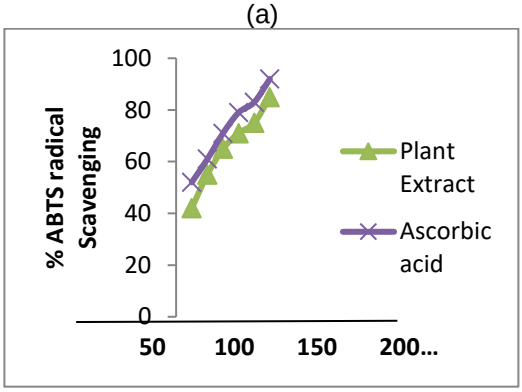
Sodium molybdate reduction activity

M. italica extract furnished valued results. The results indicate that at a concentration of 500µg/ml, the activity of molybdate radicals were 78% as compared to control 95% as seen in (Figure 3d). The IC_{50} for molybdate activity was 101µg/ml.

Polyphenolic flavonoids present in medicinal plants contain various phenolic hydroxyl groups and arise as glycosides. The presence of phenolic hydroxyl groups in flavonoids attributed to its strong antioxidants scavenging potential of free radicals (Cao et al. 2009). Current studies discovered that bioactive polyphenolic flavonoids are very essential for diminishing oxidative stress. Literature review indicated that the antioxidant properties of other effective plants such as *Mentha spicata, Plumbago indica, Teucrium polium* subsp. *Polium* and *Micromeria graeca* (Elmastas et al. 2005; Choudhury et al. 2006; Kiselova et al. 2006, Bashir and Kumar, 2020, Benali et al. 2021) were also studied for diminishing oxidative stress, which were similar in action to *M. italica* methanolic extract.



(a)



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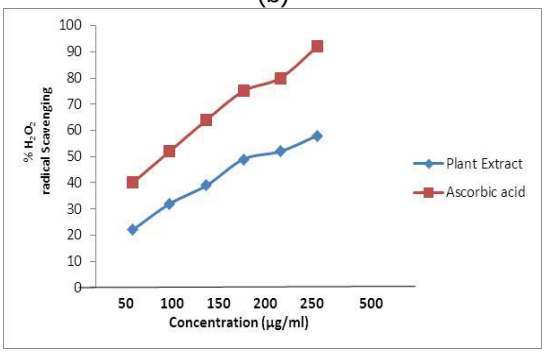
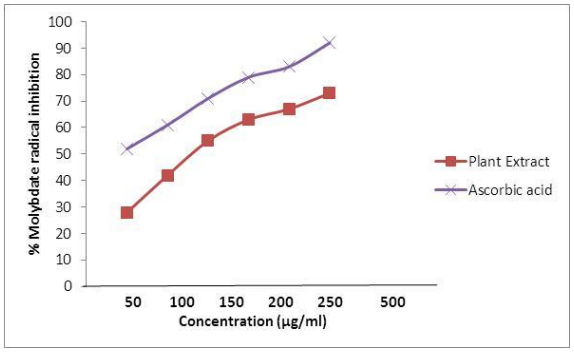


(c)



(d)

Figure 3: (a) % DPPH Scavenging (b) Interaction of crude with ABTS radical (c) Reduction of H_2O_2 (d) Reduction of sodium molybdate free-radicals.

Antidiabetic Property

α-amylase inhibitor efficiency of *M. italica*

α-amylase inhibitory activity of *M. italica* was analyzed at various concentrations (200-1000µg/ml). Of all the extracts tested, highest percentage of inhibition (70%) was found at 1000µg/ml. The Glimepiride was taken as a standard antidiabetic. Its concentration in the test was equivalent to that of the extract. Glimepiride exhibited 98% inhibition at 10µg/ml with 1.3µg/ml IC_{50} (Table 4a).

α-glucosidase inhibitor efficiency of *M. italica*

A dosage reliant increase in %age inhibition of α-glucosidase was observed in *M. italica.* At 1000 µg/ml, maximum %age inhibition was detected (87%) with 250µg/ml Concentration of Glimepiride was kept 2-10 µg/ml as standard drug for inhibitory activity of α-glucosidase. The 2µg/ml dose showed an inhibitory %age of 60 and 96% inhibition was recorded for the uppermost dose at 10µg/ml. The IC_{50} value was 1.7µg/ml. The dose dependent increase is represented in (Table 4b).

The negative effects of diabetes cause massive oxidative impairment by means of the production of ROS, followed by various diabetic complications. Insulin acts as a primary contributor to manage glucose concentration. Insulin deficiency or absence specifies very low or even no carbs metabolism. The management of diabetes is still a challenge to the health community. Earlier studies suggested that inhibition of α-amylase and α-glucosidase would suspend breakdown of carbohydrate, that in turn, would result in minimum glucose absorption and it will cause reduction in blood glucose level (Lhoret and Chiasson, 2004)

CONCLUSION

The results of the current study scientifically validate the inhibitory capacity of *M. italica* against pathogenic microbes and free radicals. The natural antioxidants may serve as therapeutic agents in avoiding or slowing a variety of health problems. The future plan for the present study includes the isolation of bioactive compounds and their characterization for the production of pharmacological products.

CONFLICT OF INTEREST

All the authors have no conflict of interest.

ACKNOWLEGEMENT

This study has been taken in the Laboratory of Biotechnology, University of Science and Technology Bannu, Pakistan.

AUTHOR CONTRIBUTIONS

SU and NG designed and performed the experiments. FD and IU wrote the manuscript. IU and SUK contributed to statistical analysis. NA and FD reviewed the manuscript. All authors read and approved the final version.

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