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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF METHANOLIC EXTRACTS OF INDIGENOUS GARLIC CULTIVARS FROM PAKISTAN

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Abstract:

In the present study, the objective was to evaluate the antioxidant and antimicrobial activities of methanolic extracts of different indigenous cultivars of garlic. The antioxidant activity of all the extracts was determined using two complementary methods, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and Hydrogen peroxide (H_2O_2) scavenging assay. All the cultivars exhibited antioxidant activity to different extent. Hazro cultivar indicated highest antioxidant activity both by DPPH assay and H_2O_2 scavenging assay as compared to other cultivars. Antimicrobial activity of the garlic extracts was also evaluated by using the agar dilution method against different microbial strains. The Pink local cultivar was most resistant to Staphylococcus aureus and Streptococcus viridians. while Desi cultivar was resistant against Escherichia coli and Clostridium septicum and Hazro cultivar was most resistant against Pasteurella multocida.

Key words: *Garlic, indigenous, methanolic extract, antioxidant activity, DPPH, H_2O_2, antimicrobial activity, Pakistan.*

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INTRODUCTION:

Garlic (*Allium sativum* L.) is the member of the family Alliaceae [1]. It is cultivated as a small crop throughout Pakistan. It is widely used for flavoring in cooking, but it has also been found useful as a medicine for the treatment of a wide range of diseases, including hypertension, cardiovascular diseases, diabetes, rheumatism, digestive disorders, etc. [2]. Garlic is an antioxidant and has been reported to reduce the risks of blood clots, stroke, stomach and colon cancer. Antioxidants are very popular among the nutrients because they have the ability to prevent many physiological diseases caused by oxidation of food material. They are known for their very important role against free radicals or Reactive Oxygen Species (ROS) in body defense system. These ROS are produced by the activity of normal cells as byproducts during aerobic metabolism.

A sufficient antioxidant status can be maintained by increasing the intake of dietary antioxidants and this can normalize the physiological functions of the living cells. Many diseases which are caused by oxidative stress can be prevented by the dietary antioxidants. Garlic has been long known to have antioxidant activity due to the presence of sulfur and polyphenols [3]. Scientists believed that synthetic antioxidants like BHA and BHT should be replaced by natural antioxidants as it has been reported that synthetic antioxidants contain toxic substances [4].

Garlic has also been known to have antibacterial activity for a long time [5]. It has high concentration of sulphur containing compounds (allicin, allin and agoene), volatile oils, enzymes (allinase, peroxidase, and microcynase) and flavonoids (quercetin, allistatin I and allistatin II), which are responsible for the defense against many bacterial strains [6][7]. Polyphenols and Quercetin are the major compounds present in garlic extract, responsible for antioxidant activity [8]. Total phenolic content and DPPH scavenging activity are positively correlated [9].

The antibacterial activity of pure crystalline Allin in the presence of raw alinase has been studied against gram positive and gram-negative bacteria [10]. Food borne bacterial pathogens are sensitive to allicin present in garlic extract. It has an inhibitory effect on the growth of many bacterial species [11]. Indu *et al*. [12] reported that garlic agents are very effective against many antibiotic resistant bacterial strains.

The aim of this study is to compare the potential antioxidant and antibacterial properties of six different indigenous cultivars of garlic from all the provinces of Pakistan in order to evaluate their medicinal potentiality and their future industrial uses.

MATERIALS AND METHODS

Sample Collection

Six different indigenous garlic cultivars were collected from all over Pakistan i.e., Hazro from Attock (A), Chinese Cultivar from Lahore (B), Desi from Kasur (C), Pink Local from KPK (D), Silver Skin from Sindh (E), and White from Balochistan (F).

Sample Preparation and Extraction

Garlic cloves from all the cultivars were peeled and washed with distilled water. For sample preparation, 100 g cloves of each garlic sample were taken. Drying process was made easy by cutting the cloves into small pieces by knife and then spreading them on paper at room temperature for 4-5 days as mentioned by Khamsah *et al.*, [13]. After drying fine powder of each garlic sample was made by crushing the dried cloves and then the powder was dipped in methanol (extracting solvent) overnight, continuously agitating at electric shaker. Then each sample was filtered by Whattman filter paper I and filtrate was collected for extraction. For extraction Soxhlet protocol by Siddhuraju *et al.* [14] was followed. Filtrate was transferred to round bottom flask and it was assembled in Soxhlet apparatus which was kept running at 60℃ for 10 – 12 hours or until all the extracting solvent removed. Moist extract was obtained by this method which was subjected to rotary evaporator for extracting solution removal. Finally the remaining methnol was removed by freeze drying and the samples were kept at 4℃ until use.

Antioxidant Activity

For the DPPH and Hydrogen peroxide scavenging assay four different concentrations of garlic extracts of six cultivars were tested i.e., 0.5, 1.0, 2.0 and 4.0 mg/ml.

a. DPPH assay

DPPH solution (0.1 M) in methanol was prepared for DPPH scavenging assay. Two ml of DPPH solution was mixed with 1ml of different concentrations (0.5, 1.0, 2.0 and 4.0 mg/ml) of each garlic extract and change in color was observed after mixing the extract in solution which showed that scavenging activity of garlic has started. The test solution was kept at room temperature for 30 minutes and then placed in spectrophotometer for absorbance at 517nm [15] [16] [17]. For negative control DPPH solution alone was used. The whole procedure was performed in triplicates and inhibition percentage was calculated by following formula.

Effect of scavenging (%age Inhibition)=[1-Xsample (517nm) /X control (517nm)]×100
X= Absorbance

b. Scavenging assay for Hydrogen peroxide

The protocol of Woisky and Salatino, [18] was followed for Hydrogen peroxide Scavenging assay. Hydrogen peroxide solution (2 mM) was made in standard Phosphate buffer pH 7.4. One ml of different concentrations (0.5, 1.0, 2.0 and 4.0 mg/ml) of each garlic extract dissolved in methanol was mixed with 0.6 ml of Hydrogen peroxide solution. Absorbance was recorded by spectrophotometer after 10 minutes at 230 nm. For control, only Hydrogen peroxide in phosphate buffer was used without any garlic sample. The percentage inhibition of different extracts at different concentrations was calculated by the following formula and compared with ascorbic acid (standard).

%age scavenging = (Control A –Sample A) / Control A × 100

A= Absorbance

Antibacterial Activity

Antibacterial activity of all six garlic cultivars was checked agains six strains of bacteria, including *Staphylococcus aureus, Clostridium septicum, Streptococcus viridans, Pasteurella multocida, Acinetobacter spp* and *Escherichia coli* using agar well diffusion method.

Agar well diffusion method

Sterile nutrient broth was prepared for the growth of bacterial strains. Ten ml of nutrient broth was taken in test tubes and each bacterial strain was inoculated in it separately and incubated for 8 hours at 37℃ and then inoculated on nutrient agar plates. Nutrient agar plates were prepared by pouring 20 ml nutrient agar medium in petri plates, wells were made by using sterile cork borer in each agar plate. Then 100µL of each extract (10, 20 and 40 mg/ml) of each of the six cultivars of garlic was poured into separate wells in agar plates. These plates were then incubated at 37℃. After 4-7 days the diameter of inhibition zones was measured.

RESULTS

In the present study, antioxidant activity of six different indigenous cultivars of garlic was evaluated by DPHH assay and Hydrogen peroxide scavenging assay. The antibacterial activity of these cultivars was also observed against three gram positive, *Streptococcus viridans, Clostridium septicum* and *Staphylococcus aureus* and three gram negative bacterial strains, including *Escherichia coli, Acinetobacter spp* and *Pasteurella multocida.*

Antioxidant Activity

DPPH assay

Ascorbic acid was used as standard antioxidant to draw the standard curve as shown in figure 1. It was observed that percentage inhibition showed an increase in order with the increase in the concentration of tested garlic extracts as given in Figure 2. Overall higher DPPH scavenging activity was recorded for cultivar Hazro i.e., 11.43, 34.16, 56.73% and 78.98 at 0.5, 1.0, 2.0 and 4.0 mg/l respectively (Figure 2). While at a concentration of 1.0 mg/ml, cultivar Silver skin showed more percentage inhibition (35.73) as compared to other cultivars (Figure 2). Least inhibition for all concentrations was recorded for cultivar Pink local i.e., 7.46, 22.18, 42.73 and 55.62% at 0.5, 1.0, 2.0 and 4.0 mg/l respectively. Overall, the DPPH scavenging activity was found to be in the order of: Hazro > Silver Skin > White > Desi > Chinese > Pink Local.

Hydrogen peroxide scavenging assay

According to Figure 3, cultivar Hazro exhibited highest percentage inhibition among all the six garlic cultivars (16.65, 42.45, 63.73 and 77.65 at 0.5, 1.0, 2.0 and 4.0 mg/ml respectively). Cultivar Pink local showed least inhibition percentage, i.e., 10.91, 32.74, 50.51 and 58.32 at 0.5, 1.0, 2.0 and 4.0 mg/ml respectively. At concentration 0.5 mg/ml cultivar Silver skin gave the highest value of inhibition (17.51%) as compared to other 5 cultivars (Figure 3). Overall, the Hydrogen peroxide scavenging activity was found to be in the order of: Hazro > Silver Skin > Desi > White > Chinese > Pink Local.

Antimicrobial Activity

The extracts of garlic cultivars were evaluated for *in vitro* antibacterial activity against gram negative (*P. multocida, Acinetobacter spp* and *E. coli*) and gram positive bacterial strains (*S. viridans, , C. septicum* and *S. aureus*) indicating different zones of inhibition (Table 2). Our results revealed that Pink local cultivar showed significantly higher inhibitory activity against two gram positive bacterial strains tried that are *S. aureus* (34.3 mm) and *S. viridians* (32.3 mm). at concentration of 40mg/ml. While Hazro cultivar at concentration of 40 mg/ml showed higher activity against *C. septicum* (25.3) and *P. multocida* (27.7 mm), Desi cultivar against *E. coli* (32.7 mm) and cultivar Silver skin for *Acinetobacter spp* (14.3 mm) as compared to other cultivars.

It can be seen from Table 2 that all six cultivars showed least activity against *Acinetobacter spp*. The present investigation has shown that all the cultivars have responded significantly against the bacterial species used in this investigation.

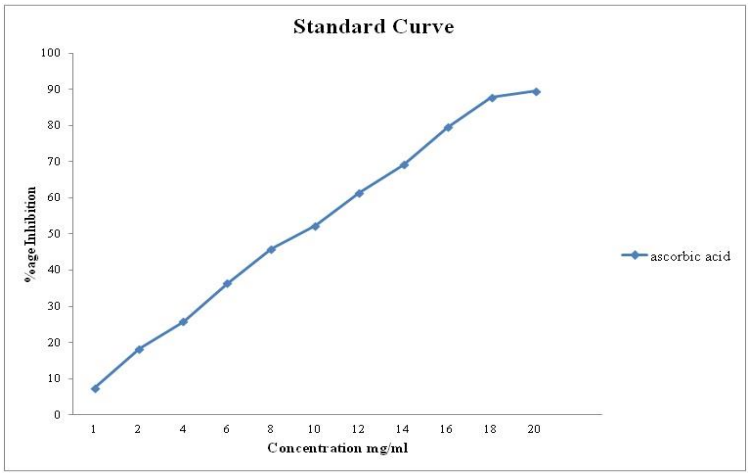

Figure 1: Standard calibration curve of ascorbic acid for determination of antioxidants

Garlic Cultivars	Concentration (mg/ml)	DPPH Assay %age Inhibition	H_2O_2 Assay %age Inhibition
Hazro	0.5	11.43±1.00^{h}	16.65±1.51^{j}
	1.0	34.16±0.99^{e}	42.45±0.85^{g}
	2.0	56.73±1.55^{e}	63.73±1.47^{d}
	4.0	78.98±0.58^{a}	77.65±1.63^{a}
Chinese	0.5	8.45±0.59^{h}	14.54±0.69^{j}
	1.0	26.85±0.95^{f}	37.57±0.67^{h}
	2.0	46.34±1.38^{d}	50.42±0.90^{f}
	4.0	58.79±1.05^{bc}	61.95±1.51^{d}
Desi	0.5	9.83±1.08^{h}	15.63±0.90^{j}
	1.0	26.75±1.39^{f}	39.54±0.54^{gh}
	2.0	46.34±0.93^{d}	58.64±0.83^{e}
	4.0	58.79±1.41^{bc}	76.59±1.52^{ab}
Pink Local	0.5	7.46±0.93^{h}	10.91±1.16^{k}
	1.0	22.18±1.46^{g}	32.74±1.43^{i}
	2.0	42.73±1.00^{d}	50.51±1.54^{f}
	4.0	55.62±2.71^{c}	58.32±0.63^{e}
Silver Skin	0.5	10.54±1.65^{h}	17.51±0.54^{j}
	1.0	35.73±1.52^{e}	40.19±1.07^{gh}
	2.0	55.89±1.48^{c}	61.45±0.53^{de}
	4.0	75.56±2.50^{a}	73.75±1.41^{bc}
White	0.5	9.75±1.41^{h}	16.05±0.56^{j}
	1.0	23.43±1.60^{fg}	39.16±0.98^{gh}
	2.0	46.31±1.21^{d}	58.43±0.66^{e}
	4.0	61.98±0.57^{b}	71.74±1.01^{c}

Mean ± standard error. Values sharing same letters differ non-significantly according to Duncan's new multiple range test (P>0.05)

Comparisons among different treatments are made by Analysis of Variance (ANOVA

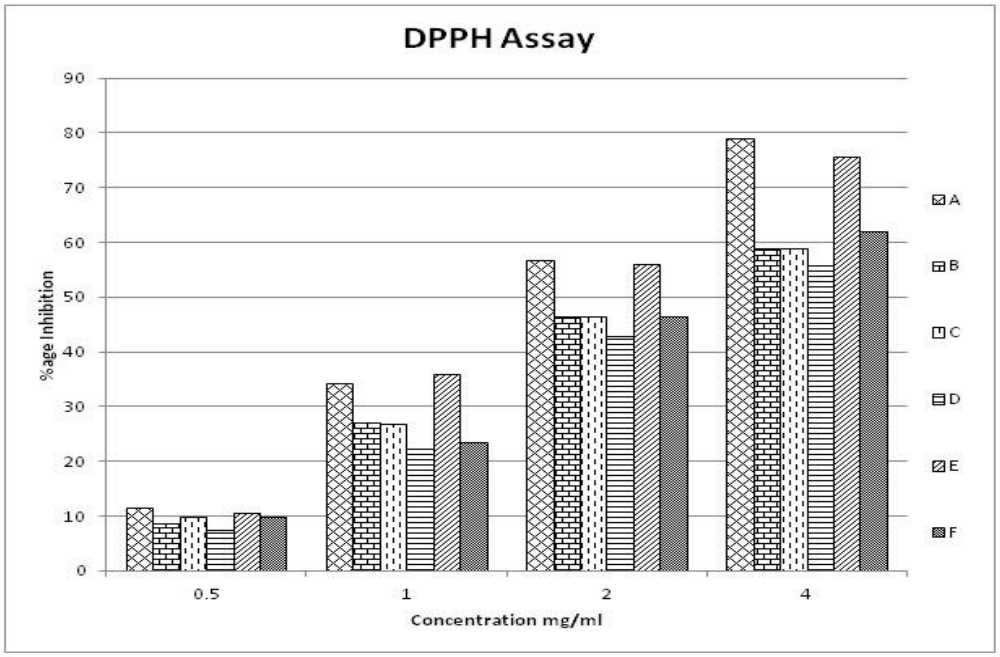

Figure 2: Antioxidant acticity of Six *Allium sativum* cultivars using DPPH scavenging assay
Legends: A. Hazro; B. Chinese; C. Desi; D. Pink local; E. Silver skin, F. White

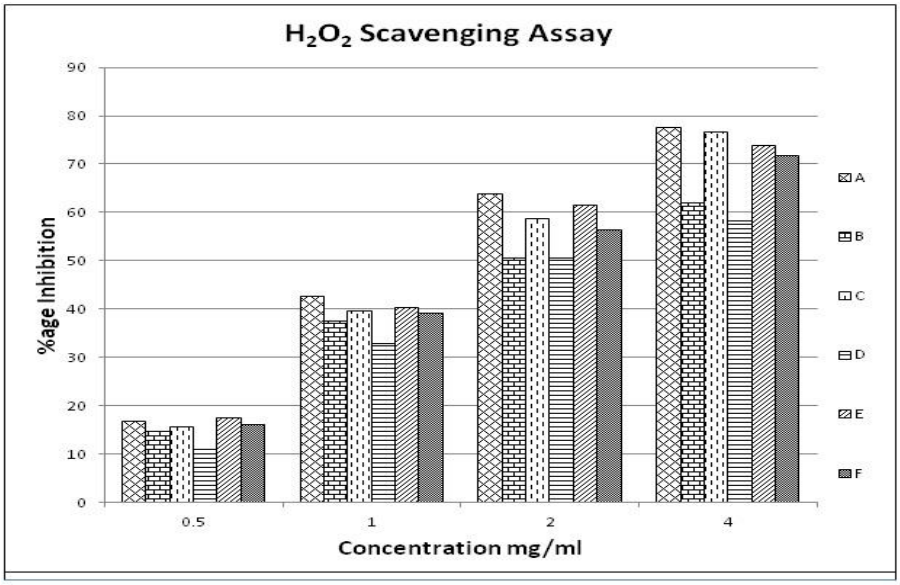

Figure 3: Antioxidant acticity of Six *Allium sativum* cultivars using Hydrogen peroxide scavenging assay.
Legends: A. Hazro; B. Chinese; C. Desi; D. Pink local; E. Silver skin, F. White

Table 1: Antibacterial Activity of six different indigenous garlic cultivars from Pakistan against six bacterial strains

Garlic Cultivars	Concentrations (mg/ml)	Zone of Inhibition (mm)					
		Staphylococcus aureus	*Streptococcus viridans*	*Clostridium septicum*	*Escherichia coli*	*Pasteurella multocida*	*Acinetobacter spp*
A	10	10.0±0.56^{m}	13.7±0.58^{n}	9.7±0.58^{l}	11.7±1.09^{n}	12.7±1.16^{j}	-
	20	16.3±0.43^{i}	21.3±0.63^{h}	16.7±0.66^{h}	19.7±1.14^{i}	17.3±0.52^{f}	9.7±0.53^{g}
	40	23.7±0.63^{d}	31.3±0.63^{b}	25.3±0.55^{a}	30. 3±2.37^{c}	27.7±1.29^{a}	11.3±1.14^{d}
B	10	9.3±0.69^{o}	11.7±2.82^{p}	9.3±0.60^{m}	10.7±1.55^{p}	8.3±0.69^{n}	-
	20	13.3±1.21^{k}	16.7±1.15^{l}	12.7±0.60^{j}	18.7±0.55^{j}	11.7±1.22^{k}	9.7±0.99^{g}
	40	22.7±1.74^{e}	26.3±1.73^{e}	22.3±0.85^{c}	27.3±1.22^{f}	17.3±0.51^{f}	13.7±1.29^{b}
C	10	9.7±1.21^{n}	10.7±0.60^{q}	9.3±1.09^{m}	14.7±0.51^{l}	11.3±1.18^{l}	-
	20	12.3±1.78^{l}	18.7±1.61^{k}	13.7±1.09^{i}	23.7±0.53^{h}	16.3±1.17^{g}	9.3±0.76^{h}
	40	19.3±1.09^{h}	25.3±1.21^{f}	20.3±0.45^{d}	32.7±1.21^{a}	25.7±0.56^{b}	12.7±0.56^{c}
D	10	16.3±1.09^{i}	15.7±1.15^{m}	10.3±1.55^{k}	11.3±1.21^{o}	10.7±0.55^{m}	-
	20	22.7±1.09^{e}	23.7±1.09^{g}	17.3±1.74^{g}	18.7±0.57^{j}	14.3±1.67^{h}	-
	40	34.3±1.73^{a}	32.3±0.57^{a}	23.3±1.18^{b}	29.3±1.66^{d}	25.3±0.53^{c}	10.3±1.18^{f}
E	10	12.3±0.51^{l}	10.3±1.84^{r}	9.7±1.15^{l}	13.3±1.67^{m}	10.7±1.17^{m}	-
	20	19.7±0.51^{g}	20.3±1.32^{j}	12.7±0.63^{j}	24.3±1.15^{g}	13.3±1.27^{i}	10.3±0.53^{f}
	40	26.3±0.84^{c}	28.3±1.21^{d}	19.3±1.09^{e}	31.3±1.07^{b}	24.7±1.03^{d}	14.3±1.07^{a}
F	10	13.7±1.84^{j}	13.3±1.09^{o}	9.3±1.66^{m}	11.7±1.13^{n}	11.3±1.03^{l}	-
	20	21.7±1.09^{f}	20.7±0.58^{i}	13.7±0.63^{i}	17.3±0.46^{k}	14.3±1.65^{h}	-
	40	30.3±1.21^{b}	29.7±1.03^{c}	18.7±1.27^{f}	28.3±1.67^{l}	23.3±0.77^{e}	10.7±0.56^{e}

Mean ± standard error. Values sharing same letters differ non-significantly according to Duncan's new multiple range test(P>0.05)

Comparisons among different treatments are made by Analysis of Variance (ANOVA

Legends: A. Hazro; B. Chinese; C. Desi; D. Pink local; E. Silver skin, F. White

DISCUSSION:

Garlic contains phenolic compounds which are responsible for the antioxidant activity of this plant. For the DPPH and Hydrogen peroxide scavenging assay four different concentrations of garlic extracts of six cultivars were tested. DPPH is a very rapid, convenient and reliable way to check the antioxidant activity of any solid or liquid food material. Antioxidant activity strongly depends on extraction solvent [19]. When DPPH solution is added to the test material the change in colour from purple to pale yellow appears in less than ten minutes which represents the scavenging activity of that material. In our present study, cultivar A (Hazro) showed the highest scavenging activity by DPPH assay, while cultivar B (Chinese cultivar) showed the least activity. Benkeblia [20] reported that garlic extracts react faster and are more effective for DPPH scavenging assay as compared to other *Allium* species.

Chung [21] worked on garlic and garlic extracts and reported that they have antioxidant activity and provide protection against Reactive Oxygen Species (ROS) in the living system. Nuutila *et al.,* [22] reported that garlic has H_2O_2 scavenging property due to the presence of phenolic compounds as they are positively correlated. Our results are in accordance with Narendhirakannan and Rajeswari, [23] which showed that the garlic bulb extracts were very potent and the H_2O_2 scavenging activity was in increasing order as the concentration of extract was increased.

The present investigation has shown that all the cultivars have responded significantly against the bacterial species. Garlic has antimicrobial activity due

to Allicin and Allin compounds which can inhibit the total synthesis of RNA and partial syntheses of DNA and proteins during bacterial cell division [24]. The growth of *S. aureus* and *S. viridians* was inhibited by Pink local cultivar at a higher rate as compared to others. Cultivar Hazro inhibited the growth of *C. septicum* and *P. multocida* more than any other cultivar but it was least resistant against *Acinetobacter spp.* Desi cultivar was most resistant against *E. coli*. Chinese cultivar showed the minimum zone of inhibition against one gram positive and two gram negative bacterial strains i.e., *S. aureus*, *E. coli* and *P. multocida.*

The growth of *S. aureus* was inhibited by cultivar Silver skin more efficiently as compared to other bacterial strains. Khashan [25] reported that garlic extract was effective against the growth of *E.coli* and *S. aureus* and this is due to allicin compound present in the garlic extract. Moderate level of inhibition was shown by cultivar White against all the gram positive and gram negative bacterial strains. According to Onyeagba and his colleague [26] it is clear that garlic extract at a concentration of 15-60 mg/ml can cause inhibition of bacterial growth. The concentration of garlic extract varies in different studies as Sivam *et al.*, [27] observed inhibition with 30 mg/ml of extract. This may be attributed to genetic diversity [28]. Yousufi [29] investigated the antibacterial activity of *A. sativum* and found that garlic is highly resistant to *E. coli* (24mm) *S. aureus* and some other bacterial species.

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