



ASSESSMENT OF PHENOLIC COMPOUNDS, ANTIMICROBIAL ACTIVITY AND FREE RADICAL SCAVENGING POTENCY OF THREE SELECTED VEGETABLES

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ABSTRACT

Phytochemicals are compounds derived from plants that are assumed to have defensive role against certain disease. They have antioxidants, anticancer, antimicrobial, antifungal, antiviral, antithrombic and anti-inflammatory properties. They have a high specificity to boost the immune system and play important role in the metabolism of hormones. The current study is based on qualitative and quantitative evaluation of total phenolics contents, phenolic compounds, antioxidant potential, free and bound phenolic acids in selected vegetables available at the local market of Hyderabad, Sindh, Pakistan. Two different extraction procedures ultrasonic-assisted base hydrolysis extraction and sonication extraction were used. Total 13 phenolic compounds were found and quantified by high-performance liquid chromatography (HPLC), in which Ferulic acid was quantified in a higher amount of 16.71 mg/g in bitter gourd. Total phenolic contents were determined by using Perkin-Elmer lambda UV/Visible spectrophotometer and higher concentration was found in Bitter gourd 92.56 mg 100/g as compared to Luffa and Brinjal with 79.03 and 66.56 mg 100/g, respectively. The antioxidant activity (DPPH assay) was measured at λ_{max} of 517 nm, results revealed that Bitter gourd possessed the higher antioxidant activity with 182.61 $\mu\text{Mol/g}$ followed by Luffa and Brinjal with 112.94 and 82.96 $\mu\text{Mol/g}$. The total Flavonoid contents were higher in Brinjal with 44.32 mg g^{-1} whereas Luffa and Bitter gourd possess the Flavonoid concentration in the range 38.02 and 34.64mg g^{-1} respectively, the total tannin contents also higher in Brinjal 31.40 mg/g followed by in Luffa and Bitter gourd with 25.17 and 21.19 mg/g respectively. Antimicrobial activity showed that, all the extracts are the highly effective against *S. aureus* as compared to *E. coli*. Finally, it is concluded that all the selected vegetables are very good sources of Phenolic compounds as well as phytochemicals and should be included in the daily human diet for good health. On the basis of obtained results, it is also suggested that these samples will be further investigated for the determination of fatty acids by GC-MS and liquid chromatography-mass spectrum (LC-MS).

Keywords: antimicrobial activity, free radical scavenging (DPPH), HPLC-DAD, phytochemical

INTRODUCTION

The term vegetable typically includes consumable part of herbaceous plant, root, stem, leaves, flower, or fruits. Herbaceous are the non-woody plants that are cultivated in farms, home garden, kitchen gardens, gathered from backwoods tree and markets for the use of house (Eme-Okafor, 2016). Vegetables are the good source of fiber contain naturally low/no in fat/cholesterol and calories. They are rich in minerals and vitamins, phenolic acids, flavonoids, and glucosinolates, which act as antioxidants, blood sugar and blood pressure influencing substances. Vegetables contain various phytochemicals which have very

important effects including, anticarcinogenic, antiviral, cholesterol-lowering, antifungal, antibacterial, antiinflammatory or antithrombotic (Jaiswal *et al.*, 2011). However ever most of these functional compounds have wide ranges of these properties. For example, antioxidative phytochemicals are phenolic compounds, saponins, protease, inhibitors, carotenoids, sulfides, phytoestrogens, monoterpenes, and phytosterols (Duyn and Pivonka, 2000; Tomas-Barberan and Espin, 2001). The European Prospective Study of Cancer (EPIC) and World Cancer Research Fund/American Institute for Cancer Research, the Health Education Authority (UK), the German Nutrition Society, and the German Cancer Society suggested that an inclusion of 50g and 375gm of vegetable in daily food intake lead to reduction in the 20% of

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the premature death and healthy life (Khaw *et al.*, 2001). It is also recommended that, the daily food intake should contain functional compound that are rich in the phytochemicals in conjugation with non-alcoholic and fat consumption (Boeing *et al.*, 2004). Recently, It was reported that, various crop production practices were used to enhance the phytochemical content and composition in many vegetables and fruits (*Vaccinium spp*) (Eichholz *et al.*, 2012; Schreiner, 2005). Hu *et al.* (2004) reported that, the mechanisms of natural antioxidant are not efficient therefore, use of antioxidant in the diet is important. These antioxidant are also widely used in the food industry to increase the shelf life of food specially those foods which are rich in the polyunsaturated fat (Isabelle *et al.*, 2010). In addition, these antioxidant are also used in the processed food to prevent the lipid oxidation (Ayaz *et al.*, 2008; Andarwulan *et al.*, 2010).

Formerly, it was revealed that, the chemicals which are synthesized in the laboratory are highly costly and toxic. Moreover due to increase in the awareness of consumer about feed additives, the information about safety, and natural source of antioxidant must be mentioned on product (Zhou and Yu 2006; Dasgupta, 2007). It is reported that, growth conditions and genotypes are the evidence to alter the different properties including phytochemicals, antioxidant and antibacterial properties (Stangeland *et al.*, 2009). From last two decades, plants and vegetable are the only natural source of phytochemical which pays attention of scientist to explore their chemical composition by using different methods and techniques. The present work also based on the same phenomena i.e in this work we determine phytochemicals analysis of selected local vegetables, their antimicrobial activity, and radical scavenging activity.

MATERIALS AND METHODS

Chemicals, standards and vegetables

Fresh three selected vegetables Bitter gourd (*Momordica charantia*), Luffa (*Luffa acutangular*), and Brinjal (*Solanum melongena*) were collected from the local market of district Hyderabad Sindh, Pakistan during April and May 2020. All chemicals and reagents used in this research work were pure analytical grade.

Processing of vegetable material

The collected vegetables were washed with tap water and then distilled water, to remove dust particles and dried in shade for 2 weeks, after drying

each vegetable ground separately with an electric grinder and kept in different glass storage bottles that have clamp lids before analysis.

Phenolic compounds evaluation

Ultrasonic-assisted base hydrolysis extraction method was used in order to extract the phenolic compounds, 0.5g of powdered vegetables was hydrolyzed with 10ml of base solution (0.372g EDTA, 2N sodium hydroxide and 1g of ascorbic acid) in 100ml polypropylene tubes by purging nitrogen (N₂) preceding covering. At that point, covered tubes were vortexed and sonicated for 30 minutes at 56°C. After hydrolysis, the samples were cooled and acclimated to hydrochloric acid (pH 2.5), and the phenolic mixture were taken out with 5x 2ml of acidic ether (ethyl acetate C₄H₈O₂) and then vortexed for 25-30 followed by centrifuged at 5,000 rpm for 10 minutes to separate the whole, aqueous layers and higher organic layer of phenolic compounds. After the solidification of phenolics compounds, they were dried by removing nitrogen followed by 5ml of 80% methanol was added. Finally, the solution was filtered via 0.45 µm syringe filters before HPLC investigation. The comparative concentrates were likewise utilized in favor of assessment of total phenolic contents (TPC) and antioxidant activity (Memon *et al.*, 2013).

Assessment of total phenolic contents (TPC)

The quantification of total phenolic contents (TPC) assay was carried out by Folin-Ciocalteu (FC) reagent method with slight modification (Memon *et al.*, 2012; Boakye *et al.*, 2015). The reaction mixtures consist 0.2ml of diluted extracts, 0.8ml of Folin-Ciocalteu (FC) reagent and 2ml of sodium carbonate (Na₂CO₃) Saturated solution (7.5%). Finally, mixtures were filled with deionized water and adjusted the volume up to 7ml then for the completion of the reaction placed the mixtures in darkness at ambient temperature for 2 hours. After incubation time absorbance was measured on Perkin Elmer lambda 35, UV/Vis spectrophotometer at 765nm. The total phenolic content was calculated from a standard, the obtained results of various extracts were expressed as Gallic acid equivalents (mg/g).

Assessment of total Flavonoid contents

Total flavonoid contents (TFC) were analyzed by reported method (Laghari *et al.*, 2011). The reaction mixture was sonicated extract contains

2ml of sodium nitrite (5% w/v), 0.5ml AlCl₃ (10%w/v) and 3ml NaOH (4.3% w/v) and distilled water up to 10ml. After that, the solution was placed in the dark for 15 mins for completion of the reaction (Laghari *et al.*, 2011), and the absorbance was measured on UV/VIS spectrophotometer, Perkin Elmer lambda 35, at 500 nm, 80% aqueous methanol was taken as reference, Rutin was used as a standard with the concentration range from 1-100 µg/ml The results were expressed as rutin equivalents (Rutin eq. µg/ml) and predicted from a standard curve.

Evaluation of total tannin contents

In brief, 0.2g powder samples of each vegetable were extracted in 1% hydrochloric acid in methanol for 20 mins at 30°C then 1ml of extract and 5ml of vanillin reagent (50:50 mixture of 1% vanillin 8% HCl in methanol) was treated for 20 mins at 30°C (Siddiqui *et al.*, 2017), and the absorbance was measured on UV/VIS spectrophotometer, at 500nm. HCl (4%) as a substituent of vanillin reagent was added to the extract and used as a blank. While catechin was used for standard. The results were estimated by the standard curve of catechin which obtained in the concentration range 6.25 µg/ml to 200 µg/ml (Siddiqui *et al.*, 2017).

Radical scavenging activity (RSA)

Two ml of 0.1 mmol of DPPH solution was mixed to 2mL sonicated extract of every vegetable followed by shaking and placed in the dark for up to 25 minutes. The quercetin standards were prepared, and the range of concentration was used 1 to 10 µmol. Subsequently, the absorbance was determined through a UV/Vis spectrophotometer at 517nm. The antioxidant activity equivalent to quercetin was adapted from the standard curve and expressed as µmol/100g of the sample (Memon *et al.*, 2013; Amron and Konsue, 2018).

Identification and Quantification of phenolic compounds by (HPLC-DAD)

HPLC Thermo Finnigan, SCM 1000 (California, USA) connected with a DAD (diode array detector) system was used for the analysis of phenolic compounds. Reverse-phase Hypersil Gold C-18 (Thermo Corporation, USA) was used to separate different phenolic compounds, (Memon *et al.*, 2013). The gradient mobile phase solvent 0.1% formic acid in water (A) and in methanol (B), were used. The rate of flow was kept as 1 ml/min and the gradient linear was 5% to 30% for 25 min, followed by 30% for 45 min,

gradient linear elution was altered as of 30% up to 100% for 52 minutes. After 52 min, elution was carried back to 5% for 3 min in order to equilibrate the column. Furthermore, with the help of DAD, the UV analysis was done, set at 270, 310, and 325nm. The software Chromquest Version 4.2 was applied for data interpretation. All phenolic compound structures were separately confirmed by comparison their UV spectra and retention time (tR) with related standards. For the construction of each standard curve, the standard solutions in the different concentration series from 1-50 µg/ml were introduced into the HPLC-DAD system. The concentrations of the different phenolic constituents were calculated through peak area. In accordance with their individual standard curves.

Determination of antimicrobial activities

The antibacterial action of vegetables was determined by the strategy depicted by (Rahman *et al.*, 2017). Four concentrations of 125, 250, 500 and 1000 µg mL⁻¹ were set up in 100% DMSO (dimethyl sulfoxide), and DMSO was utilized as the negative control. Whatman No. 1 paper with 20µL of different obsessions (100% diluted) was arranged outwardly of the petri dishes place in an incubation center for 24 hours at 37°C. After that, the antibacterial movement estimating by the territory of hindrance width in mm around the circles and determined MIC (minimum inhibition concentration) values (Bouchekrit *et al.*, 2016).

RESULTS

Twenty diverse phenolic guidelines (aldehyde, protocatechuic, vanillin, pyrogallol, ferulic, *m*-coumaric, 2,4,6-trihydroxy benzoic, *p*-coumaric, gallic acid, gentisic, β -resorcinolic, *p*-hydroxybenzoic, sinapic, vanillic, chlorogenic, hypogallic, caffeic, protocatechuic, *o*-coumaric, syringic, and, cinnamic acid) were run into a chromatographic segment of spectra frame work SCM 1000 instrument, with flow rate 1.0 mL/min and, spectra were recorded at 325, 310 and 270nm. Every sample had a three replicate (Table 1). The HPLC profiling results revealed that, the presence of thirteen phenolic compounds i.e. Gallic acid, sinapic acid, Catechin, chlorogenic acid, Naringin vanillin, vanillic acid, caffeic acid, *p*-hydroxybenzoic acid, ferulic acid, *m*-coumaric acid, *p*-coumaric acid, and cinnamic acid, were identified as bound phenolic compounds from selected vegetables which are given in (Table 2).

Table 1. Identification and Separation of phenolic standards with respective linearity and retention time by HPLC coupled with diode-array detector

Standards	t _R (min)	λ _{max} (nm)	R ²	Regression equation
Gallic acid	7.69	227, 272	0.999	y = 305726x - 249684
2,4,6-THBA	9.51	216, 255, 292	0.998	y = 49119x + 29082
Protocatechuic acid	13.16	228, 259, 294	0.997	y = 530511x + 112990
Pyrogallol aldehyde	14.18	234, 291	0.999	y = 337860x + 147020
Protocatechuic aldehyde	14.35	234, 281	0.998	y = 548015x + 303632
Naringinin	14.92	232, 327	0.999	y = 13444x - 1829.4
Vanilline	17.26	233, 281, 307	0.999	Y=626260x-138097
Sinapic acid	19.92	255,294	0.991	y = 643555x - 1E+06
β-resorcinolic acid	18.99	255, 294	0.998	y = 200138x + 46398
Hypogallic acid	19.61	232, 314	0.998	y = 82657x - 14787
Vanillic acid	27.19	223, 260, 294	0.999	y = 289390x - 82077
Caffeic acid	28.73	233, 323	0.995	y = 169059x - 140031
Chlorogenic acid	32.83	217,233, 327	0.998	y = 97008x - 33773
Syringic acid	33.33	225, 275	0.999	y = 214749x - 72422
Naringin	34.8	232,283, 327	0.998	y = 37927x + 35542
P-HBA	35.18	234, 308	0.999	y = 88856x - 14995
p-coumaric acid	40.45	232, 309	0.995	y = 213962x - 333316
Ferullic acid	46.79	235, 322	0.998	y = 174006x + 127640
m-coumaric acid	47.59	216, 232, 278	0.999	y = 533000x + 78590
o-coumaric acid	48.75	232, 277, 330	0.999	y = 7E+06x - 2E+06
Cinnamic acid	49.05	230, 280, 330	0.992	y = 568487x + 305505
Rutin	62.18	257, 285, 355	0.999	y = 14303x - 77826
Gentisic acid	63.8	235, 287	0.999	y = 13791x - 40728

t_R= Retention time**Table 2.** Bound phenolic acids profiling from selected vegetables

Phenolic acids	t _R (min)	Bitter gourd Mg/g ± RSD	Luffa Mg/g ± RSD	Brinjal Mg/g ± RSD
Gallic Acid	8.7	5.53 ± 0.13	5.65 ± 0.12	2.02 ± 0.05
Naringrin	15.75	9.29 ± 0.12	3.15 ± 0.11	2.45 ± 0.14
Vanilline	20.18	ND	5.77 ± 0.13	ND
Sinapic Acid	23.71	4.95 ± 0.02	6.82 ± 0.11	1.52 ± 0.01
Vanillic Acid	25.18	ND	3.66 ± 0.09	ND
Catechein	27.39	ND	ND	2.11 ± 0.12
Chlorogenic Acid	29.34	ND	ND	ND
Caffeic Acid	32.98	7.47 ± 0.12	ND	4.56 ± 0.16
P-HBA	35.18	2.43 ± 0.01	14.57 ± 1.02	ND
P-Coumaric Acid	45.50	6.21 ± 0.11	ND	11.26 ± 1.21
m-Coumaric Acid	47.59	ND	2.17 ± 0.04	ND
Cinamic Acid	49.05	ND	ND	ND
Ferulic Acid	51.16	16.71 ± 0.23	ND	5.78 ± 1.11
TBPA		52.59	41.79	29.70

t_R= Retention time. RSD= Relative standard deviation, ND= Not detected

Figure 1 (A) is the chromatogram of bitter gourd extract in which 7 phenolic compounds were identified. These compounds were Gallic acid (5.53mg/g), Naringrin (9.29 mg/g), sinapic acid (4.95mg/g), Caffeic acid (7.47mg/g), p-HBA determined in lowest amount as (2.43mg/g), p-coumaric acid (6.21mg/g), and ferulic acid obtained in highest amount as (16.71mg/g) at retention time 8.7, 15.75, 23.71, 32.98, 35.18, 45.50 and 51.16 minutes respectively.

Figure 2 (A) is the chromatogram of luffa extract in which 7 phenolic compounds were identified. These compounds were Gallic acid (5.65mg/g), Naringrin (3.15mg/g), Vanillin (5.77mg/g), sinapic acid (6.82mg/g), Vanillic Acid (3.66mg/g), p-HBA determined in highest amount as (14.57mg/g), and m-Coumaric Acid obtained in low as (2.17mg/g) at retention time 8.7, 15.75, 20.18 23.71, 25.18 35.18 and 47.59 minutes respectively.

Figure 3 (A) is the chromatogram of brinjal extract in which 7 phenolic compounds were identified. These compounds were Gallic acid (2.02mg/g), Naringin (2.45mg/g), sinapic acid determined in lowest amount as (1.52mg/g),

Catechin (2.11mg/g), Caffeic acid (4.56mg/g), *p*-coumaric acid obtained in highest amount as (11.26mg/g), and ferullic acid (5.78mg/g) at retention time 8.7, 15.75, 23.71, 27.39, 32.98, 45.50 and 51.16 minutes respectively.

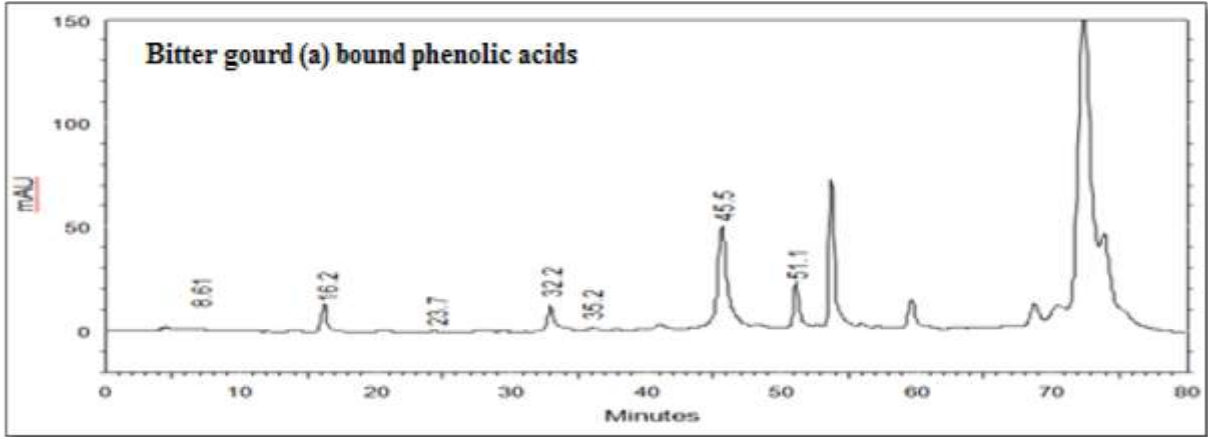


Figure 1. (A) HPLC chromatogram of bitter gourd

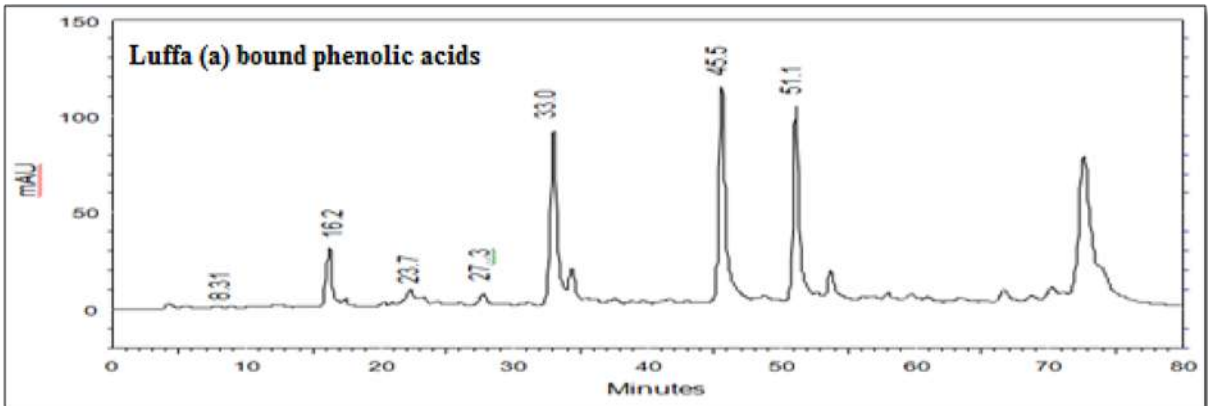


Figure 2. (A) HPLC chromatogram of luffa

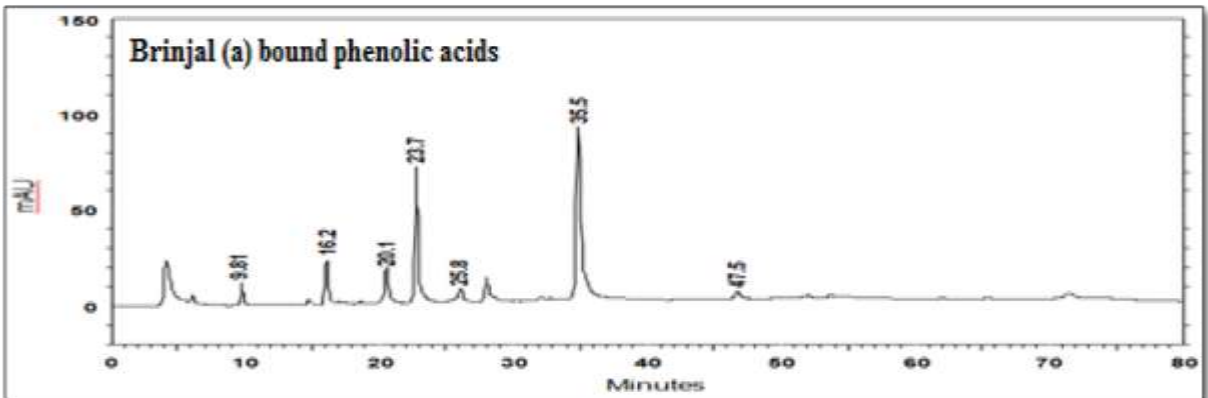


Figure 3. (A) HPLC chromatogram of brinjal

Total Phenolic, Free radical scavenging activity, total flavonoid, and total tannin content of vegetables are shown in Table 3. The highest total phenolic contents (TPC) are quantified in bitter gourd as 92.56mg/g and lowest was in brinjal as 66.56mg/g luffa possess the average value which is 79.03 mg/g, the radical scavenging activity (RSA) was also greater in 182.61 $\mu\text{mol/g}$ and smallest was in brinjal as 82.96 $\mu\text{mol/g}$ again luffa possesses the average value of RSA as 112.61 $\mu\text{mol/g}$, total flavonoid contents (TFC) are highest in brinjal which is 44.32 mg/g and smaller determined in bitter gourd as 34.64 mg/g, TFC was 38.02 mg/g in luffa and total tannin contents (TTC) also find higher in brinjal 31.40 mg/g and lowest in bitter gourd as 21.19 mg/g and luffa possess 25.17 mg/g.

Table 3. Total phenolic content, anti-oxidant activity, total flavonoids content, and total tannins content

Samples	T.P.A (mg/g)	RSA ($\mu\text{mol/g}$)	T.F.C (mg/g)	T.T.C (mg/g)
Bitter gourd	92.56	182.61	34.64	21.19
Luffa	79.03	112.94	38.02	25.17
Brinjal	66.56	82.96	44.32	31.40

TPA= Total phenolic acids, RSA= Radical scavenging activity, TFC= Total flavonoid contents, TTC= Total tannin contents

Table 4. Antibacterial activities of bitter gourd

Microorganism strains	Concentration in $\mu\text{g/mL}$					MIC Value ($\mu\text{g/mL}$)
	1000	500	250	125	Control	
<i>Escherichia coli</i>	16	8	3	0	-	250
<i>Staphylococcus aureus</i>	18	10	6	2	-	125

MIC = Minimum inhibition concentration

Table 5. Antibacterial activities of luffa

Microorganism strains	Concentration in $\mu\text{g/mL}$					MIC Value ($\mu\text{g/mL}$)
	1000	500	250	125	Control	
<i>Escherichia coli</i>	12	5	2	0	-	250
<i>Staphylococcus aureus</i>	15	8	5	2	-	125

MIC = Minimum inhibition concentration

Table 6. Antibacterial activities of brinjal

Microorganism strains	Concentration in $\mu\text{g/mL}$					MIC Value ($\mu\text{g/mL}$)
	1000	500	250	125	Control	
<i>Escherichia coli</i>	8	5	2	0	-	250
<i>Staphylococcus aureus</i>	11	6	3	1	-	125

MIC = Minimum inhibition concentration

Evaluation of antibacterial activity

The methanol extract of selected vegetables were tested against the two different microbes are present in Tables 4, 5 and 6. The result revealed that the bitter gourd, Luffa and Brinjal shows excellent activity against *S. aureus* at 18, 10, 6, and 2, 15, 8, 5, and 2 and 11, 6, 3 and 1 $\mu\text{g/ml}$ at concentration range 1000, 500, 250,

and 125 ppm were individual. These extracts (vegetables) also show the enhanced activity against *E. coli*, with activity as 16, 8, and 3, 12, 5 and 3, and 8, 5 and 2 $\mu\text{g/ml}$ for bitter gourd, Luffa and Brinjal at concentration 1000, 500 and 250 ppm whereas the selected vegetables shows zero activity at 125 ppm. The (MIC) against *E. coli* 250 and for *S. aureus* was 125 $\mu\text{g/ml}$ determined. As a comparison study, the extract of bitter gourd revealed high activity and minimum show in brinjal respectively.

DISCUSSION

These vegetables are found to be rich sources of phenolic compounds, flavonoid and tannin contents, in quantitative analysis. The classification of phenolic content (mg/g) lies as given bitter gourd > luffa > brinjal ranging from 92.56 to 66.56 (mg/g). Phenolic compounds are known as regular antimicrobial (Raju and Rao, 2012). Tannins have additionally been perceived because of their remedial potential. The most elevated measure of tannins was accounted for in brinjal (31.40 mg/g). Leafy foods contain various kinds of phenolic compounds in various degrees; go about as potential antioxidant and a vital aspect for saving health and keeping away from various diseases (Krinsky and Johnson, 2005). Beforehand, in comparative investigations (Aqil *et al.* 2006; Ramya *et al.* 2011) similar values for the Total Phenolic Contents of methanol (74.33 \pm 5.13 mg/g) and ethanolic extracts (4.9 mg/g) were reported. Contrasts between the findings might be because of different conditions like temperature, area, environment, diseases and bug openness inside the species, selection of parts tried a season of taking examples, and techniques for declaration (Singh *et al.*, 2014).

CONCLUSION

It concluded that ultra-sonic assisted base hydrolysis extraction is the most efficient method for the extraction, one can get maximum yield. The selected vegetables are very good sources of phenolic compounds, they are highly effective against microorganisms and oxidants because they contain a wide and broad range of significant organic compounds which highly beneficial for good health. It is also concluded that selected vegetables need much more attention from researchers to assess their leftover wellbeing impacts by deciding unsaturated fats, minerals, and heavy metal contamination by using different analytical techniques.

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AUTHOR'S CONTRIBUTION

J. Mangi: Main author of manuscript

A. Jat: Carry all practical work

N. Soomro: Assessed anti-microbial activity of selected vegetables

A. J. Pirzada: Identification of micro-organism

A. R. Sidhu: Performed all chemical analysis of vegetables

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