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RESEARCH ARTICLE

Evaluation of Phytochemical Screening, Antimicrobial and Antioxidant Activities of Ethanol Extracts of *Cucumis flexouses* and *Cucumis reticulatus* Seeds

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The current study is designed to investigate the phytochemical screening and to examine the potential antimicrobial, antioxidant activities of ethanol extract of *Cucumis flexouses* and *Cucumis reticulatus*. The ethanol extract of *C. flexouses* and *C. reticulatus* seeds were subjected to preliminary phytochemical screening for the confirmation of various phytochemicals and their total phenolic and flavonoid content is verified by the colorimetric method. Subsequently, antimicrobial activity of both extracts at the concentration of (25, 50, 100 mg/ml) against various microbial organisms was evaluated via the disc diffusion method by measuring zone of inhibition and estimating minimum inhibitory concentration (MIC). The antioxidant activity was assessed by ferric thiocyanate (FTC) and DPPH free radical scavenging method. The ethanol extract of *Cucumis flexouses* showed maximum antibacterial activity against gram-positive bacteria including *Staphylococcus aureus* (29.0±0.05mm), *Bacillus subtilis* (17.0±0.02 mm) followed by gram-negative bacteria *Escherichia coli* (22.0±0.05 mm) and *Pseudomonas aeruginosa* (15.04±0.34mm) respectively at 100mg/ ml concentration as compared to standard drug. Moreover, both the extracts showed no activity against fungal species *Candida albicans*. Similarly, significant antioxidant effects at 100mg/ml concentration (1.17±0.025) were also observed by FTC and (1.09±0.017) by DPPH method. The result of the current study depicts that ethanol extract of *C. flexouses* seeds possess significant in-vitro antimicrobial and antioxidant effects as compared to *C. reticulatus* seed extract and could be a good substitute for many infectious diseases.

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INTRODUCTION

Antibiotics play an important role in the cure of various infections, for example tuberculosis, pneumonia and meningitis (Chen *et al*., 2019). However, due to the emergence of resistance against numerous microbial organisms by antibiotics, the death ratio by infectious diseases is increasing globally. Hence, the demand of new cost effective and easily accessible antibiotics, which can target multi resistant strains of bacteria is increased (Golkar *et al.*, 2014; Wright, 2014; Lim *et al*., 2020).

The *Cucurbitaceae* family of plants commonly known as gourd family is comprised of about 130 genera and 800 species of numerous edible plants and fruits. The fruits of this family contain a rich quantity of seeds having numerous nutritional and therapeutic properties. According to researchers, seeds of the plants provide balanced nutrition in terms of ingredients (Souri *et al.*, 2019). They contain biologically active constituents and are a good source of protein, fiber and other minerals, also these are the chief source of vegetable oil; but unfortunately, in spite of their employment in medicine, they are usually discarded and unused (Seema *et al.*, 2017).

Biological analysis has proven various potential pharmacological activities of the seeds extracts of fruits and vegetables belonging to this family such as antioxidant, anticancer, antihypertensive, hypoglycemic,

cardioprotective, antilipidemic, antimutagenic, antiapoptosis, anti-inflammatory and antihelminthic effects (Mercy *et al*., 2011; Morais *et al.*, 2017).

Thorough literature review with respect to these fruits indicated scarcity of research work on seeds of these fruits. Qualitative as well as quantitative analysis of seed extracts of these fruits confirmed the presence of rich phytochemical compounds in seeds. Inspired by the therapeutic importance of *Cucurbitaceae* family, current study was especially designed to investigate the in-vitro antimicrobial and antioxidant properties of ethanolic seed extracts of *Cucumis flexouses* (Snake melon) and *Cucumis reticulatus* (Galia melon).

MATERIALS AND METHODS

Collection and identification of Plant: *Cucumis flexouses* (Snake Melon) and *Cucumis reticulatus* (Galia Melon) fruits were purchased from local market, Imtiaz super store, Karachi, Pakistan. Seeds were authenticated and recognized by Dr. Muneeba Khan, Taxonomist in Botany Department, Karachi University. The issued voucher specimen no (95600) and (95607) respectively was deposited in Centre for Plant Conservation, Herbarium and Botanic Garden, University of Karachi

Preparation of seed extract: Seeds was carefully separated from the fruit of *C. flexouses* and *C. reticulatus* by hand, cleansed and washed to remove any adhering residue. Afterwards, the seeds were air dried under the shade and pulverized to coarse powder (particle size 500 µm) by electric blender (Moulinex, France). Seeds in the powder form were soaked in ethanol (powder to solvent ratio was 1:10 w/v) at 37-40°C temperature for 24hr along with stirring at various time intervals. After that, solvent was rinsed with cotton cloth and further filtrated by (Whatman no .1) filter paper. Later on, ethanolic extracts was vaporized by reducing pressure at (45-50°C) in rotary evaporator, followed by freeze drying at -20°C. The final extracts yield was semisolid dark brown residue of *C. flexouses* (19.24%) and *C. reticulatus* (18.34 %). Extracts were stored in amber brown colored air tight sterile bottles and kept at -4°C in refrigerator until used.

Preliminary Phytochemical screening

Qualitative analysis: Phytochemical screening of seed extracts of *C. flexouses* and *C. reticulatus* was performed to investigate the presence of major phytoconstituents i.e. alkaloids, flavonoids, glycosides, cardiac glycosides, phytosterols, phenolic compounds, resins, steroids, saponins, terpenoids, triterpenoids and tannins according to the standard methods (Sony *et al.*, 2011; Sateesh *et al.*, 2012; Parbuntari *et al.*, 2018).

Test for alkaloids (Wagner's test): 2 ml of each extract was dissolved in 1% dilute hydrochloric acid and then filtered. The filtrate was treated with Wagner's reagent (Iodine in potassium Iodide). Appearance of reddish-brown precipitate confirmed the presence of alkaloids.

Test for flavonoids: 1 ml of each extract was mixed with lead acetate (10%) solution. The appearance of yellow precipitate confirmed the existence of flavonoids.

Test for Glycosides: The extract of each plant was hydrolyzed with (HCl) and then neutralized with (NaOH) solution. A few drops of Fehling's solution A and B were added. The appearance of red precipitate indicates the presence of glycosides.

Test for Phytosterols: 1ml of each extract was combined with acetic anhydride (2ml) + H_2SO_4 (2ml). The violet colour changed to blue, designates the presence of sterol.

Test for Phenolic compounds: One drop of $FeCl_3$ was added into each extract showed intense violet color which indicates phenolic compounds.

Test for Resins: 2ml of chloroform and 10ml of acetic anhydride added in both extract and then dissolved by gentle heating. 0.5ml of H_2SO_4 was added after cooling. The bright purple color appeared which indicated the presence of resins.

Test for Steroids (Liebermann Burchard reaction): 200mg of each extract was added with 10ml of chloroform. 2ml of this filtrate reacted with 2ml of acetic anhydrides and conc. H_2SO_4. The formation of a blue-green ring indicates steroids' presence.

Test for Saponins: 1ml stock solution of each extract diluted with 20 ml of distilled water in a test tube. It was shaken with a hand for 15 minutes. The appearance of the foamy lather layer indicates the presence of saponins.

Test for Terpenoids (Salkowski test): Each extract (5ml) mixed with chloroform (2ml) and add 2ml of conc. H_2SO_4 in it. Appearance of reddish-brown color confirmed the existence of terpenoids.

Test for Triterpenoids: Each extract (1 mL) was mixed with chloroform (2ml) and add acetic anhydride (1ml). Afterward, conc. H_2SO_4 (1ml) was added in this solution.The appearance of reddish violet color confirmed the presence of triterpenoids.

Test for Tannins: Each extract (0.5gm) was boiled in water (10ml) in test tube. After filteration, 0.1% $FeCl_2$ was added, brownish green color confirmed the presence of tannins.

Quantitative analysis of seeds extract

Total phenolic compounds: The total phenolic compounds were verified by employing the Folin-Ciocalteu reagent. Each extract at the amount of 100mg dissolved in 100ml was dissolved in Folin-Ciocalteu reagent (750ml) and with saturate sodium carbonate (750ml). The absorbance was recorded at 765nm, after 90 minutes with the help of a UV-vis spectrophotometer (Beckmen DU640) (Pellegrini *et al*., 2017).

Total flavonoids compounds: The total flavonoids compound was verified by adding 1.5ml of 2% aluminum chloride ($AlCl_3.6H_2O$) solution into an equal volume of both extracts. After vigorously shaking the absorbance of

the solution at 367nm was recorded by UV- vis spectrophotometer.

Antimicrobial activity

Test organisms: Five micro-organisms (*Pseudomonas aeruginosa* (MTCC 26932), *Staphylococcus aureus* (MTCC 26934), *Escherichia coli* (MTCC 27630), *Bacillus subtilis* (MTCC 27629) and *Candida albicans* (MTCC 8933) were used as a test organism, obtained from the Microbiology Department of Karachi University, Microbial Type Culture Collection Center (MTCC), and stored at -20°C. The bacterial and fungal isolates were grown for 20hr at 35°C in Nutrient agar (Himedia) and Sabourad dextrose agar (Himedia) respectively and stored at 4°C until utilized.

Standard Antimicrobial drugs: Standard antimicrobial agents Streptomycin (0.4 mg/ml) and Nystatin (50,000 I.U/ml) were used as a positive control group.

Evaluation of Antimicrobial activity by Disc diffusion method: Antimicrobial potential of seeds extracts of *C. flexouses* (CFE) and *C. reticulatus* (CRE) was evaluated by Agar well diffusion method using Muller Hinton Agar (MHA) medium. Agar well diffusion method is generally used to evaluate the antimicrobial activity of plant extracts. For this each culture plates were seeded with test organism then permitted to stabilize. Then holes (5.0 mm in diameter) were made on seeded agar with sterile cork borer. Aliquot of 0.05 ml of each seed extract was filled in open well and for the proper diffusion allowed to stand for 1 hour, later incubated at 37°C for 24hr. Diameters of zone of inhibition were recorded in millimeter which indicates the susceptibility of seed extract against microorganisms. (Ette *et al.*, 2017).

Minimum inhibitory concentration (MIC): Serial dilutions of the extract at various concentrations were prepared to determine inhibitory concentration. The lowest concentration of the extract that showed minimum inhibition of growth was recorded and taken as minimum inhibitory concentration (MIC) (Bahar *et al.*, 2018).

Antioxidant activity: Antioxidant activity of three different concentrations (100 mg/ml, 50 mg/ml and 25 mg/ml) of CFE and CRE was analyzed via in vitro antioxidant assessment by ferric thiocyanate method and DPPH scavenging method. All values of assay were in three repetitions and means of these is considered.

Ferric thiocyanate (FTC) method: This method is used to determine the amount of peroxide formation that reacts with the ferrous chloride (FeCl2) pigment; resultant decreased peroxide concentration indicates antioxidant activity. The sample mixture in test tube was consisted of extract (0.5ml), linoleic acid emulsion (2.5 ml), 2 ml potassium phosphate buffer (0.05M, pH 7.0) and phosphate buffer (0.05 M, pH 7.0). Afterwards sample mixture was incubated in darkness at 37°C; subsequently peroxide level was measured by spectrophotometer (thermo scientific) at 500 nm. Ascorbic acid was used as positive control (Abdel-Gawad *et al*., 2019).

DPHH Free radical scavenging assay: The free radical scavenging activity of CFE and CRE was directly measured by DPPH (1,1-diphenyl-2-picrylhydrazyl). This method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant. Different concentrations of sample 0.1ml were mixed with 3.0ml of ethanolic DPPH (60µM) solution. The mixture was kept in dark for 25min and absorbance was measured by spectrophotometer (thermo scientific) at 517nm. Ascorbic acid was used as reference standard drug. The following equation was used to measure the percentage inhibition.

Percentage inhabitation (%) = Absorbance of control – Absorbance of test /Absorbance of control × 100

Statistical analysis: The data of current investigation were statistically analyzed by using SPSS 23.0 version. All values are the mean from three repetitions. Data were analyzed by ANOVA at ($P<0.05$) significance level.

RESULTS

Phytochemical screening: Preliminary phytochemical screening of ethanol extract of *C. flexouses* seeds and *C. reticulatus* revealed the presence of all major phytoconstituents such as alkaloids, flavonoids, glycosides, phenols, tannins, triterpenes, saponins as shown in Table 1. All phytochemicals were detected in both seed extracts except Sterol and triterpenoids which were not detected in CRE. However, the quantitative analysis of ethanol extract of *C. flexouses* and *C. reticulatus* seeds indicates the presence of the highest concentration of total phenolic content in *C. flexouses* (8.98±0.45 mg GAE/g) and (6.45±0.65mg QE/g) of flavonoids content as compared to *C. reticulatus* extract (4.68±1.03 mg GAE/g) phenolic content and (0.89±1.54 mg QE/g) of flavonoid content (Fig. 1).

Antimicrobial assessment: The present investigation revealed that both extracts possess a varying potential of antimicrobial activity in dose depended manner by disc diffusion method against various test organisms as shown in Table 2. Ethanol extract of *C. flexouses* seeds showed more significant antibacterial activity and their susceptibility in order to inhibit microbial growth as follows; *Staphylococcus aureus* (29.0±0.05 mm) >*Escherchia coli*.

Table 1: Qualitative phytochemicals screening of seeds extracts of *C. flexouses* and *C. reticulatus*

Phytochemical	*C. flexouses* extract (CFE)	*C. reticulatus* extract (CRE)
Alkaloids	++	++
Flavonoids	++	+
Glycosides	++	++
Phenolic compounds	++	+
Phytosterols	+	-
Resin	++	+
Steroids	+	+
Saponins	++	+
Terpenoids	++	+
Triterpenoids	++	-
Tannins	++	+

Presence of phytochemicals (+), phytochemicals in high concentration (++), absence of phytochemicals (-).

Table 2: Antimicrobial activity of *C. flexouses* and *C. reticulatus* seeds extracts

Bacterial Strain	Extract Conc. (mg/ml)	Diameter of zone of growth inhibition (mm)			
		C. flexouses extract (CFE)	*C. reticulatus* extract (CRE)	Streptomycin 0.4mg/ml	Nystatin (50,000 I.U/ml)
Bacillus subtilis	100	17.0±0.02	8.9±0.06	24.0±0.23	
	50	9.01±0.31	3.5±0.01	-	
	25	2.51± 0.02	0.91±0.34	-	
Staphylococcus aureus	100	29.0±0.05	19.0±0.02	34.0±0.07	
	50	17.0±0.14	13.0±0.05	-	
	25	9.56±0.34	3.1±0.12	-	
Escherichia coli	100	22.0±0.05	9.0±0.31	40.0±0.07	
	50	9.01±0.67	3.1±0.5	-	
	25	3.7±0.51	1.3±0.2	-	
Pseudomonas aeruginosa	100	15.04±0.34	5.12±0.07	37.0±0.78	
	50	8.031±0.45	1.3±0.02	-	
	25	2.54±0.01	0.21±0.3	-	
Candida albicans	100	-	-	-	22±0.01
	50	-	-	-	-
	25	-	-	-	-

The data of all values are the mean of three replications. (-) show no activity.

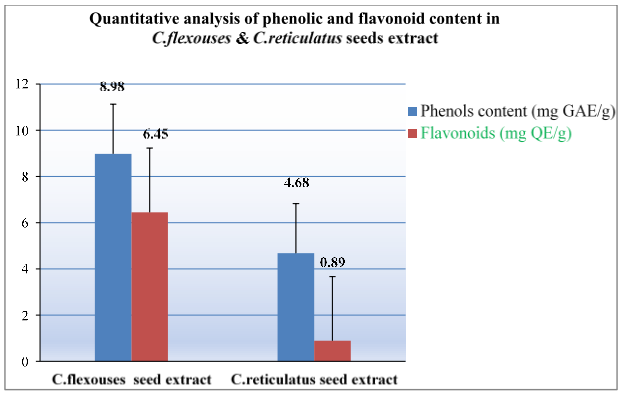

Fig. 1: Concentration of phenolic and flavonoids compound in *C. flexouses* and *C. reticulatus* extract.

Table 3: Minimum inhibitory concentration (mg/ml) of *C. flexouses* and *C. reticulatus* seeds extracts

Bacterium Isolate	Extract Gradient Conc. (mg/ml)	
	C. flexouses (CFE)	*C. reticulatus* (CRE)
Bacillus subtilis	22.35±0.15	35.03±0.24
Staphylococcus aureus	17.10±0.01	30.04± 0.21
Escherichia coli	32.01±0.03	38.30±0.34
Pseudomonas aeruginosa	29.0± 0.14	35.10 ±0.51

The data of all values are the mean of three replications.

Table 4: Antioxidant activity of ethanol seed extracts of *C. flexouses* and *C. reticulatus* by FTC method

Ethanol extracts	% inhibition		
	25mg/ml	50mg/ml	100mg/ml
C. flexouses extract	0.56±0.031	0.813±0.012	1.17±0.025
C. reticulatus extract	0.03±0.024	0.69±0.034	0.96±0.021
Ascorbic acid	0.81±0.017	1.34±0.021	2.60±0.041

The data of all values are the mean of three replications.

Table 5: Antioxidant activity of ethanol seed extracts of *C. flexouses* and *C. reticulatus* by DPPH free radical scavenging activity

Ethanol extracts	%inhibition		
	25mg/ml	50mg/ml	100mg/ml
C. flexouses extract	0.46±0.021	0.614±0.030	1.09±0.017
C.reticulatus extract	0.02±0.013	0.19±0.014	0.86±0.046
Ascorbic acid	0.79±0.017	1.34±0.021	2.60±0.041

The data of all values are the mean of three replications.

(22.0±0.05 mm) >*Bacillus subtilis* (17.0±0.02 mm) > *Pseudomonas aeruginosa* (15.04±0.34 mm) respectively at 100 mg/ml. However, ethanol extract of *C. reticulatus* seeds exhibited less significant antibacterial activity against *Staphylococcus aureus* (19.0±0.02 mm) >*Escherichia coli* (9.0±0.31mm) >*Bacillus subtilis* (8.9±0.06 mm) and *Pseudomonas aeruginosa* (5.12±0.07 mm) at 100 mg/ml concentration as compared to *C. flexouses* seeds. Conversely, less significant antimicrobial effects were observed at 50mg/ml and 25mg/ml concentration of both extracts as compared to the positive control. Moreover, both extracts showed no activity against *Candida albicans*.

Minimum inhibitory concentration: The MIC of ethanol extract of *C. flexouses* seeds; ranged from 17.10 mg/ml to 32 mg/ml. However, the MIC values of *C. reticulatus* seeds extract showed the highest value ranging from 30 to 38 mg/ml indicating comparatively less susceptibility towards bacterial organisms (Table 3).

Antioxidant activity by ferric thiocynate method: Table 4 showed antioxidant activity of *C. flexouses* and *C. reticulatus* at three different doses of extract in a dose-dependent manner. The significant antioxidant effects as presented by percent inhibition (1.17±0.025 %) was observed at 100 mg/ml of *C. flexouses* extract. However, *C. reticulatus* seed extract gave (0.96±0.021%) inhibition at 100 mg/ml.

DPPH free radical scavenging activity: The higher dose of *C. flexouses* and *C. reticulatus* exhibits significant results as shown in Table 5. The maximum radical scavenging activity was observed at 100mg/ml of *C. flexouses* extract (1.09±0.017%). However, the same concentration of *C. reticulatus* produced less significant result (0.86±0.046%).

DISCUSSION

Antibiotics are the first line treatment for many infectious diseases. However, resistance occurs frequently, because of the use of broad-spectrum antibiotics. Alternative interventions are required which may restore the diversity of microorganisms, reduce the clinical symptoms and recover patient's health (Kamath *et al.*, 2019).

The current study revealed that *C. flexouses* and *C. reticulatus* seed extract exhibits significant antimicrobial potential in a dose dependent manner as shown by diameter of zone of inhibition as compared to the standard reference drug. Higher concentration i.e. 100mg/ml

showed more significant results than lower concentration 50 mg/ml and 25 mg/ml against the same microbial test organisms. The crude extract of *C. flexouses* and *C. reticulatus* seeds contains numerous phytochemicals like tannins having ability to inactivate several enzymes, microbial linkage and weaken the cell wall of bacteria (Prosberg *et al.,* 2016). Present investigation revealed that seed extract of *C. flexouses* has more potential to inhibit the microbial growth than *C. reticulatus* at the same concentration.

Moreover, both seed extracts showed more susceptibility toward Gram positive bacteria than Gram negative bacteria. This can be due to the structure variation in cell wall. Gram negative bacteria are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances; including antibiotics (Cama *et al.,* 2019, Heesterbeek *et al*., 2019).

Phytochemical investigation of the seed extracts has indicated the presence of flavonoids and saponins (Chiejina *et al*., 2016). It can be assumed that antimicrobial activity of the extracts is due to the presence of flavonoids and saponins (Raji *et al.,* 2019).

Both the extracts also showed significant antioxidant potential assessed by both the FTC and DPHH method in a dose dependent manner as indicated by percentage inhibition by the extracts. More antioxidant activity was observed at the highest concentration i.e., 100mg/ml as compared to the lower doses. It has been found that phenolic compounds possess antioxidant activity as they have capability to donate electron and chelate metal ions (Mazid *et al.,* 2011). Similarly, tannins also work as an antioxidant, having potential ability to chelate metal ions and precipitate protons (Tortora *et al.,* 2001; Karmijit *et al.,* 2003; Ankita *et al.,* 2012). Phytochemical analysis of these plants has confirmed the presence of this phytoconstituent (Naym *et al.,* 2009; Rajasree *et al.,* 2016; Pratima *et al*., 2019). Antioxidant activity of these extracts can be attributed to the presence of these bioactive compounds.

Conclusions: The extract of *C. flexouses* and *C. reticulatus* exhibited significant *in vitro* antibacterial and antioxidant activity. This suggests that these extracts can be employed in the development of new antimicrobial drugs for the treatment of various infectious diseases. In-vivo studies on large numbers of animals should be planned in future so as to further validate the results. This study provides sufficient evidences regarding the *in vitro* antibacterial and antioxidant potential of ethanol extract of *C. flexouses* and *C. reticulatus* seeds. There is intense need to isolate the active ingredients which will help in future discovery of novel drugs.

Authors contribution: Hafiza Tuseef Sayyar perceived the idea, conducted research, write-up of manuscript and data analysis. Syeda Afroz supervised the whole project, critically examined the manuscript. Tahira Assad helped in data analysis and bibliography.

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