In vitro antimicrobial, antioxidant activity and phytochemical screening of *Apium graveolens*

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Abstract: The present study evaluates the phytochemical screenings, antioxidant activity and antimicrobial assay of *Apium graveolens* L. The phytochemical screening showed the presence of flavonoids, tannins, saponins and steroids in *Apium graveolens* while terpenoids was absent. The total phenolic content was slightly higher in methanolic fraction (63.46±12.00mg GAE/g) followed by ethanol (36.60±12.28 mg GAE/g) and hexane fractions (34.86±6.96mg GAE/g). The flavonoid content was high in methanolic extract (56.95±7.14mg Quorcetin/g) and low level of the content was found in methylated spirit extract (29.2±3.15mg Quercitin/g). Antioxidant activity assayed by FRAP was higher in methanolic fraction (12.48±1.06 mmole of $FeSO_4$ equivalent/litre of extract) compared with other extracts. Likewise, good antimicrobial activity was measured by crude ethanol fraction against *S. aureus* (MIC=0.12±0.03µg/ml) and *S. typhi* (MIC= 0.5±0.2µg/ml). Results also that ethanolic fraction was effective against *A. flavus* (MIC= 05±1.0µg/ml).

Keywords: Phyto-chemical, antibacterial activity, antifungal activity, anti-oxidant, *Apium graveolens.*

INTRODUCTION

Wild plants have played an important role for the local people in providing their basic necessities. Plants are used for the treatments of different illnesses like diabetes, infections, skin diseases etc and serve as a source of many potent drugs. Plants are used for the treatments of different illnesses and serve as a source of many potent drugs (Cowan, 1999). The different plant parts used included root, stem, flower, leaves and modified plant organs (Bakht *et al*., 2011 a, b, c and d; 2012; 2013 a,b; 2014 a, b,c; Parveen and Bakht, 2013; Nasir *et al*., 2015). The therapeutic behavior of these plants can be attributed to different bio-active compounds produced by these native plant species grown widely in different ecological regions of the world. A large number of modern drugs have been isolated from natural sources, notably of plant origin (Cowan, 1999). The investigation of such plants could be helpful in understanding their therapeutic efficiency, nutraceutical potential and plants and animal toxicity (Zavala *et al*., 2009; Zavala *et al*., 2011). Pakistan, among a very few countries is bestowed with thousands of useful plants used in foods and drugs. These plants are routinely used for common ailments through traditional knowledge. Research literature revealed that various phyto-chemicals including flavonoids, terpenoids, carotenoids, saponins, antibacterial and anti-oxidant are the main constituents of Pakistani flora.

Apium graveolens belonging to family Umbiliferaceae, locally known as Ajmod (English name Celery), is an annual medicinal plant wildly available in various parts of the KPK province of Pakistan. Celery is cultivated in the moderate mild extreme region as significant gardening crop and the leafy stems are served as popular vegetables. The seeds are used to inhibit diseases like bronchitis and liver-spleen ailments (Satyavati *et al.*, 1987). Celery is also used for the treatment of high-blood pressure, cirrhosis, hepatitis, cancer, diabetes, hyper-glycemia and regulates heart function (Sowbhagya, 2014). The Phyto-chemicals investigation of Celery have shown the presence of poly-phenols, flavonoids, steroids, tannins, saponins, tarpenoids including bio-molecules and have hepato-protective, cytotoxic, estrogenic, anti-estrogenic, anti-oxidant and anti-oxidant properties (Popovic *et al*., 2006; Ciz *et al*., 2010; Pandey *et al*., 2012; Edziri *et al*., 2012). The chemical constituents in Celery showed the analgesic, anti-inflammatory and antimicrobial effect (Momin and Nair, 2001; Misic *et al*., 2008; Garvey *et al*., 2011; Shad *et al*., 2011; Edziri *et al*., 2012).

MATERIALS AND METHODS

Plant materials

The seeds of the Celery (*Apium graveolens*) plant were purchased from the local market of Peshawar and Nowshera distract of KPK province of Pakistan. Seeds were dried at room temperature (25ºC) under shade and grinded by tissue homogenizer to fine powder (Infinigen™ Tissue Mixer Mill, ACT Gene. These powdered samples were sealed in plastic bags and stored at 4ºC until analyzed.

Extractions

The powdered plants samples were macerated in aqueous methanol, ethanol, hexane and methylated spirit (Sigma-

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Aldrich) and kept at room temperature for 7 days. The solution was stirred three times a day during this period for thorough mixing and was then filtered (Whatman™ Whatman UK). One litre each of fresh solvents were added to the seed material and filtered again through Wattman filter paper and this process was repeated thrice. The filtered solution was evaporated with the help of a rotary evaporator (Rotavapor R-R 210/R215; BUCHIL Labortechnik AG).

Phyto-chemical screening

Determination of tannins

Crude extract of seed (0.5gm) was boiled in 20ml of water and allowed to cool and filtered. About 1% ferric chloride solution was added and observed for blue black or brownish green color. The appearance of required color was the indication of the presence of tannins (Trease and Evans, 1983).

Determination of saponins

For the estimation of saponins, two grams of subject plant samples in powdered form was boiled in 20ml of distilled water in water bath for half an hour, allowed to cool and filtered. Ten ml of the filtrate was mixed with 5ml of distilled water. The mixture was constantly shaken until a persistent froth appeared. For confirmation, few drops of olive oil were added to froth and shaking was continued till the formation of emulsion (Sofowara, 1993).

Determination of flavonoids

Similarly, for the flavonoids determination crude extract of the subject plant was prepared and filtered as discussed previously and about 5ml of the dilute ammonia solution was mixed with each crude extract. Afterwards, known amount of H_2SO_4 (Conc) was added to the mixture. The confirmation of flavonoids was confirmed by the appearance of yellow color in each plant extract (Sofowara, 1993).

Determination of terpenoids

For the identification of terpenoids, a mixture containing 5ml of plant sample and 2ml of chloroform was prepared. Three ml of H_2SO_4 (Conc) was added to the mixture for the formation of a layer. Terpenoid presence was established by the appearance of reddish brown color (Harborne, 1973).

Determination of steroids

Approximately 20g of each crude plant sample was taken and soaked in ethanol in a volumetric flask and boiled for 10 minutes. The extract was filtered and the ethanol fraction was extracted and separated and the crude solid sample remained was dissolved in 3ml chloroform followed by the addition of acetic anhydride (4.5ml) and sulfuric acid (0.5ml). Steroids presence was confirmed by change of color of crude extract from violet to green (Sofowara, 1993).

Determination of total polyphenol content

FCR (Folin-Ciocalteu reagent) was used for the determination of total phenolic content in the crude fractions of subject plant with Gallic acid as a reference for comparison. Approximately 100µl of each crude fraction was mixed with about 900µl of water followed by the addition of FCR (500µl). $Na_2CO_3.10H_2O$ solution (20%) was made from this solution and 1.5ml was separated and subsequently poured into above mixture. The whole mixture was heated for 2 hours and absorbance was measured by spectrophotometer at 765 nm. Total polyphenols were then evaluated by comparing with Gallic acid as standard.

Determination of total flavonoid content

In the present study, the total flavonoid content in the subject plant was evaluated by colorimetric method using quercitin as a standard. About one milliliter of crude fraction of each plant sample was treated with 4ml of distilled water and about 300µl of $NaNO_2$ and $AlCl_3$ each were poured in this mixture. The mixture was warmed for about 5 minutes and NaOH was added in this mixture so that the volume of the mixture became10 ml. Absorbance was measured by spectrophotometer and total flavones were represented as quercitin equivalents (QE) in mg/g of dry crude extract.

Determination of antioxidant activity by ABTS protocol

The ABTS or 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) test was employed for analyzing total antioxidant potential of crude fractions of subject plant. In this method trolox was used as standard. ABTS solution was prepared that was further reacted with K. persulphate in order to get ABTS radical cation. The solution was then kept for a night after which 10µl from each crude sample was made to react with 1ml of ABTS Radical cation solution. Absorbance was measured at 734nm using spectrophotometer. All the values obtained were compared with trolox (standard).

Determination of antioxidant activity by DPPH protocol

The powdered Celary seeds were extracted with methanol for 40-48 hrs and solvent was separated through vacuum evaporator. One ml of DPPH was mixed with ethanol and poured into about 2.5ml of each crude fraction. Antioxidant activity was used measured by spectrophotometer at 515nm 30 minutes of the reaction. All readings/data obtained was compared with trolox (standard).

Determination of antioxidant activity by FRAP protocol

In this protocol the antioxidant activity of the subject plant was estimated using ferric reducing antioxidant power (FRAP) assay as described by Benzie and Strain (1999). FRAP reagent was be prepared by mixing 20 mm ferric chloride hexa hydrate, 10 mm of TPTZ (tripyridy1-

s-triazine) and 300mm acetate buffer. Plant sample was mixed with 1.9ml of FRAP reagent. This Absorbance was recorded at 593 nm through spectrophotometer.

Disc diffusion susceptibility method

The antibacterial activity of different solvent extracted samples of Celary (*Apium graveolens*) was carried by disc diffusion assay as described in Bauer *et al.* (1998) and antifungal activity by Ramdas *et al.* (1998). Different antibiotics (Ciprofloxacin at 50µg concentrations for Gram-positive and Gram-negative bacteria; 50µg Amphotercin B for fungus were aseptically placed over the seeded agar plates. The plates were incubated at 37ºC for 24 hours and the diameter of the inhibition zones

Minimum inhibitory concentration (MIC) measurements

Minimum inhibitory concentration (MIC) was measured according to Khan *et al.* (2007). Briefly, seed extracts of *Apium graveolens* was dissolved in 2ml distilled water and added with 2 drops tween-80 for complete dissolution. The suspension of each test organisms was prepared by approximately 10^7 per ml and 1 drop of this suspension was added to each broth dilution. After 18-24 h incubation at 37°C, the tubes were examined for the growth. The MIC of the extract was taken as the lowest concentration that showed no growth. Growth was observed in those tubes where the concentration of the extract was below the inhibitory level and the broth medium was observed turbid (cloudy). Distilled water with 2 drops of tween-80 and Ciprofloxacin and Amphotercin B were used as negative and positive control, respectively.

Microorganisms tested

The selected bacterial strains for the current study were *Bacilus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherchia coli* and *Salmonella typhi*. The fungal strains for the current investigation were *Trichophyton longifusus*, *Aspergillus flavus*, *Fusarium solani*, *Candida glaberata* and *Candida albicans*.

STATISTICAL ANALYSIS

The experiment was repeated in triplicate and MSTAT computer software was used for the analysis of the data. Standard deviation was calculated for each sample (Steel *et al.*, 1997).

RESULTS

Poly-phenol and flavanoid contents

The results indicated that *Apium graveolens* contained appreciable amount of tannins, flavonoids, steroids, saponins, however, terpenoids content was almost negligible. It is reported that large amount of flavonoids might be responsible for their healing effect against pathogenic microorganisms (Cowan, 1999). The subject plant was screened for total polyphenols and flavanoid (table 1). The results revealed that maximum phenolic content was present in methanolic fractions of *Apium graveolens* (63.46±12.00mg GAE/g), followed by ethanolic (36.60±12.28mg GAE/g) and hexane extracts (34.86±6.96mg GAE/g). Methylated spirit fraction had the lowest phenolic content (29.2±3.15mg GAE/g). The flavonoid content of the *Apium graveolens* was higher in methanolic extract (56.95±7.14mg Quercetin/g) and low concentration of the same constituent was noted in methylated spirit extract (29.2±3.15mg Quercetin/g).

Table 1: Total flavonoid and phenolic contents of *Apium graveolens*

Plant extracts	Total flavonoid contents	Total phenolic contents
Methanol	56.95±7.147	63.46±12.00
Ethanol	55±12.87	36.60±12.28
Methalted spirit	29.2±3.15	15.10±4.32
Hexane	34.86±6.96	25.63±5.387

±= Standard deviation

Anti-oxidant assay

Antioxidant activity was measured by three different methods for certainty. These methods included DPPH, ABTS and FRAP. When measured by DPPH, the data revealed that antioxidant activity was highest in methanolic fractions (IC_{50}: 1.41±0.270) followed by ethanolic extract (IC_{50}: 1.09±0.260) and methylated spirit extract (IC_{50}: 0.92±0.18) when compared with the standard trolox and quercetin (IC_{50}=1.05±0.05 and 0.05 respectively) (table 2). The data regarding antioxidant activity obtained through ABTS protocol revealed that ethanol fraction showed significant scavenging activity (IC_{50}=0.71±0.15) followed by methanol extract (IC_{50}= 0.49±0.06), ethanolic extract (IC_{50}=0.47±0.07) and methylated spirit extract (IC_{50}=0.42±0.09). Similarly, the standard drugs trolox and quercetin recorded ABTS value of IC_{50}= 1.08±0.14 and 0.30 respectively (table 2). Free radical scavenging activity was also determined by FRAP method. Analysis of the data revealed that all crude fractions of *Apium graveolens* exhibited anti-oxidant activity in the range of IC_{50}=8.64-12.48 (table 2). Methanolic fraction exhibited higher antioxidant activity (IC_{50}=12.48±1.06) followed by hexane fractions (IC_{50}= 10.45±0.83) and methylated spirit (IC_{50}=10.4±0.74).

Antimicrobial activity

The present study also investigates antimicrobial activity of crude fractions (methanol, ethanol, methylated spirit and hexane) of *Apium graveolens* against five each bacterial strains (*Staphylococcus aureus*, *Escherichia. coli*, *Psteropseda aeruginosa*, *Salmonella typhi* and *Bacillus subtilis*) and fungal strains including *Trchophyton longifusus*, *Candida albicans*, *Candida*

Table 2: Antioxidant potential of *Apium graveolens* using DPPH, ABTS and FRAP protocol

Plant extracts	IC_{50} value		
	DPPH	ABTS (TEAC)	FRAP* (mmol/L)
Methanol	1.41±0.270	0.49±0.06	12.48±1.06
Ethanol	1.09±0.260	0.71±0.15	8.64±0.92
Methalted spirit	0.92±0.18	0.42±0.09	10.4±0.74
Hexane	0.91±0.15	0.47±0.07	10.45±0.83
Trolox	1.05±0.05	1.09±0.14	-
Quercitin	0.05	0.06	-

*Ferric reducing activity (expressed as mmole of $FeSO_4$ equivalent/litre of extract). ±= Standard deviation

Table 3: Antibacterial assay of different crude fractions of *Apium graveolens* (MIC; average Value ±SD, µg/ml)

Plant extracts	*E. coli*	*P. aeruginosa*	*S. typhus*	*B. subtilis*	*S. aureus*
Methanol	17.7±6.58	11.16±2.05	0.96±0.1	0.82±0.28	1.11±0.50
Ethanol	0.74±0.15	0.67±0.24	0.12±0.03	1.17±0.30	0.5±0.2
Methalted spirit	0.73±0.20	10.0±1.64	0.54±0.23	0.46±0.15	0.46±0.01
Hexane	0.60±0.1	3.40±0.49	0.93±0.16	0.85±0.07	-
Ciprofloxacin	0.06	1.5	0.25	0.5	0.25

Ciprofloxacin (standard drug), ±= Standard deviation

Table 4: Antifungal assay at 5mg/ml of different crude fractions of *Apium graveolens* (MIC; average Value ±SD, µg/ml)

Plant extracts	*T. longifusus*	*C. albicans*	*C. glaberata*	*A. flavus*	*F. solani*
Methanol	65.65±7.09	39±11	32.3±19.13	50±0.1	50.0±0.15
Ethanol	50.60±5.50	25.6±8.32	35.3±6.02	05±1.0	18±3.0
Methalted spirit	0	31.3±16.4	-	-	33.6±7.02
Hexane	45.6±3.05	38±10.81	33±7.21	12±3.0	13±2.0
Amphotericine B	0.06±0.42	0.5±0	0.24±0.01	0.5±0	0.5±0

±= Standard deviation amphotericine B (standard drug)

glaberata, *Fusarium solani* and *Aspergillus flavus* (tables 3 and 4). Results indicated that all fractions of *A. graveolens* showed good activity against *Escherchia coli*, *Staphylococcus. aureus*, *Salmonella typhi*, *Bacillus subtilis* and *Pteropseda aeruginosa*. It was observed that crude ethanolic fraction of *Apium graveolens* exhibited good activity as compared to other fractions including hexane, methylated spirit and methanol. Similarly, the methanolic fraction was effective against *S. typhi* and *B. subtilis* showing MIC value of 0.96±0.1µg/ml and 0.82±0.28µg/ml respectively, while the same fraction was least effective against *E. coli* recording MIC value of 17.7±6.58µg/ml. Hexane and methylated spirit showed at par activity while methylated spirit was slightly less effective inhibition against *P. aeruginosa* (MIC=11±1.64 µg/ml) (table 3). *A. grveolens* was also evaluated against antifungal activity using different strains of fungi. The data showed that the ethanolic fraction of *A. graveolens* was more effective against *A. flavus* having MIC value (05±1.0µg/ml) followed by hexane fractions (MIC= 12±3.0µg/ml) (table 4). Similarly, methylated spirit fraction also proved effective against relevant fungal strains followed by methanolic fractions (table 6).

DISCUSSION

Our results showed that *Apium graveolens* contained appreciable amount of tannins, flavonoids, steroids, saponins, however, terpenoids content was almost negligible. It is reported that large amount of flavonoids might be responsible for their healing effect against pathogenic microorganisms (Cowan, 1999). The subject plant was also tested for total polyphenols and flavanoid (table 1). The results indicated that maximum phenolic content was present in methanolic fractions of *Apium graveolens* followed by ethanolic and hexane extracts. Methylated spirit fraction on the other hand had the lowest phenolic content. The flavonoid levels of the *Apium graveolens* were higher in methanolic extract and lower concentration of the same constituent was observed in methylated spirit extract. Yao *et al*. (2010) revealed that major phenolic acids identified in the extracts of different cultivars of celeries were caffeic acid,*p*-coumaric acid, and ferulic acid, while the identified flavonoids were apigenin, luteolin, and kaempferol. These results agree with Popovic *et al*. (2006), Kaur and Arora (2009), Ciz *et al*. (2010) and Pandey *et al*. (2012).

Antioxidant activity in the same plant specie was measured by three different assays including DPPH, ABTS and FRAP. When tested by DPPH, the results revealed that antioxidant activity was highest in methanolic fractions followed by ethanolic extract and methylated spirit extract compared with the standard trolox and quercetin (table 2). The data measured by ABTS protocol revealed that ethanol fraction showed significant scavenging activity followed by methanol extract, ethanolic extract and methylated spirit extract (table 2). Free radical scavenging activity determined by FRAP assay indicated that all crude fractions of *Apium graveolens* exhibited anti-oxidant activity in the range of IC_{50}= 8.64-12.48 (table 2). Methanolic fraction exhibited higher antioxidant activity followed by hexane fractions and methylated spirit. Popovic *et al*. (2006) reported that both the extracts of root and leaves of *Apium graveolens* are good scavengers of OH and $DPPH^{\bullet}$ radicals and reduce LPx intensity in liposomes, which points to their protective (antioxidant) activity. They also concluded that many of the investigated cultivars of celeries had high levels of phenolics and exhibited high antioxidant capacity. Similar results are also revealed by Ciz *et al*. (2010), Ediziri *et al*. (2012) and Pandey *et al*. (2012).

The present study also investigated the antimicrobial activity of crude fractions (methanol, ethanol, methylated spirit and hexane) of *Apium graveolens* against five each bacterial strains (*Staphylococcus aureus, Escherichia. coli, Psteropseda aeruginosa, Salmonella typhi* and *Bacillus subtilis*) and fungal strains including *Trchophyton longifusus, Candida albicans, Candida glaberata, Fusarium solani* and *Aspergillus flavus* (tables 3 and 4). Results indicated that all fractions of *A. graveolens* was very effective and showed good activity against *E. coli, S. aureus, S. typhi, B. subtilis* and *P. aeruginosa*. It was also noted that crude ethanolic fraction of *A. graveolens* revealed good activity as compared to other fractions including hexane, methylated spirit and methanol. Similarly, the methanolic fraction was effective against *S. typhi* and *B. subtilis* while the same fraction was least effective against *E. coli*. Hexane and methylated spirit showed similar activity while methylated spirit was slightly less effective against *P. aeruginosa* (table 3). Rani and Khullar (2004) revealed that moderate antimicrobial activity was shown by *Apium graveolens* against multi-drug resistant *Salmonella typhi*. Similar results are also reported by Bonjar (2004), Misic *et al*. (2008), Shad *et al*. (2011), Garvey *et al*. (2011) and Edziri *et al*. (2012). *A. grveolens* was also evaluated for their antifungal activity using different strains of fungi. The data indicated that the ethanolic fraction of *A. graveolens* was more effective against *A. flavus* followed by hexane fractions (table 4). Similarly, methylated spirit fraction also showed activity against relevant fungal strains followed by methanolic fractions (table 4). Momin and Nair (2001) reported that methanolic extract of *Apium graveolens* seeds inhibited the growth of *Candida albicans* and *Candida parapsilasis* at 100µgmL^{-1}. Edziri *et al*. (2012) concluded that the methanolic extract of *Apium graveolens* had the best antifungal activity against *Candida albicans, Candida kreussei* and *Candida parapsilosis* with MIC values ranging between 0.08 and 0.31mg/ml. Similar results are also reported by Misic *et al*. (2008) snd Shad *et al*. (2011).

CONCLUSION

The phytochemical screening showed the presence of flavonoids, tannins, saponins and steroids in *Apium graveolens* while terpenoids was absent. The total phenolic, flavonoid contents and antioxidant activity were higher in methanolic fraction Good antimicrobial activity was measured by crude ethanol fraction against *S. aureus, S. typhi* and *A. flavus.*

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