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Kalsoom Qureshi
Department of Agricultural Chemistry, The University of Agriculture Peshawar, Pakistan.

Saqib Jahan
Deptt. of Pharmacy University of Swabi, Khyber Pakhtunkhwa Pakistan

Neelam
Department of Agricultural Chemistry, The University of Agriculture Peshawar, Pakistan

Muhammad Amin
South China Sea Institute of Oceanology, The Chinese Academy of Sciences, Guangzhou 510301 Guangdong, P. R China

Muhammad Zain Akram
National Center for Nanoscience and Technology, Zhongguancun, Beijing, The Chinese Academy of Sciences, P. R. China

Riffat Jehan
Institute of Chemical Sciences, University of Peshawar, Pakistan.

Correspondence:
Muhammad Amin
South China Sea Institute of Oceanology, The Chinese Academy of Sciences, Guangzhou 510301 Guangdong, P. R China

Anti-Leishmanial, Anti-Fungal, Brine Shrimp Lethality, Anti-Leishmanial and Insecticidal Assay of *Apium graveolens* L available in Khyber Pakhtunkhwa-Pakistan

Kalsoom Qureshi, Saqib Jahan, Neelam, Muhammad Amin, Muhammad Zain Akram and Riffat Jehan

Abstract
Apium graveolens L belonging to the family "Umbelliferae" an unadventurous vegetable and also known to be beneficial in the treatment of a number of diseases was obtained in the month of May-July, from its natural habitat as wild species and the *Apium graveolens* seeds (Celery) from three different markets of Khyber Pakhtunkhwa i.e. Peshawar, Swat and DI Khan. During this study, the wild plant as well as the market celery is investigated for bioassays. The crude Hexane extract was screened for their lethality bioassay. The research work revealed that Hexane extract showed no brine shrimp lethality while the extract exhibited negative insecticidal activity. The result indicated that the crude Hexane extract showed strong inhibitory activity against *Trichphyton longifuss* (80%) and *Microsporum canis* (80%). Antifungal bioassay of Hexane crude extract displayed some promising results.

Keywords: *Apium graveolens* L, Bioassays.

1. Introduction
Modern medication system is principally based on synthetic chemical compounds that are often found causing harmful side effects on human body system. For this reason, the world today is focusing more and more on research in field of herbal medicine. The use of medicinal plants already plays a vital role in covering the basic health needs in developing countries, and these plants could offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms [1, 2].
The inclusion of bioactive plants and plant compounds in the feed of animals, as food components, can sometimes also provide a variety of health promoting benefits. (Durmic et al. 2012) [3] While medicinal plants have been exploited for centuries, and are still in use for treatment of humans and animals, these practices generally lack documentation, research and standardization.
The plant under study, *Apium graveolens* L., is a hapaxanthic herb that is grown annually under particular conditions. It flowers from June to August, and the seeds get ripen from August to September. Celery as an important garden crop, is cultivated widely in the temperate zones and the bleached leaf stalks are appreciated as a popular vegetable. In India, celery seeds are utilized for treatment of asthma, bronchitis and spleen diseases. Also in Indian system, the seeds of *Apium graveolens* L, are used as medicine for the treatment of liver ailments [5].
Apium graveolens Linn belongs Umbelliferrie family. The investigation for bioactive compounds in methanolic extract of *Apium graveolens* resulted in the isolation and characterization of mosquitocidal, nematicidal, and some other antifungal compounds (Momin *et al.*, 2000). An essential oil extracted from the plant was found to have a calming effect on the central nervous system while some of its constituents have antispasmodic, sedative and anticonvulsant actions. Sedative effects of the oil [7] and the central effects of various fractions of essential oil [8] have been described in literature.
Wild celery has a long history of medicinal and food use. It is an aromatic bitter tonic herb that reduces blood pressure, relieves indigestion, stimulates the uterus and is anti-inflammatory. The ripe seeds, herb and root are aperient, carminative, diuretic, emmenagogue, galactogogue, nervine, stimulant and tonic. It is used in treating rheumatism and kidney complaints. The seeds of celery contain a number of bioactive constituents such as coumarins, flavonoids, sesquiterpenoids, aromatic glucosides and phthalides [9]. Phthalides is a bioactive natural compound that occurs widely in umbelliferous plants [10].

During this study, the wild plant as well as the market celery is investigated for the presence bioactive compounds in this the herbal plant for its utilization in the field of alternative medicines to cure different diseases in man and animals.

2. Experimental Details

2.1 Sample collection: Samples of *Apium graveolens* L were obtained in the month of May-July, from its natural habitat as wild species and seeds (Celery) of the same were collected from three different Markets of Khyber Pakhtunkhwa province of Pakistan i.e. Peshawar, Swat and DI Khan.

2.2 Extraction with Organic solvents: The powdered plant material samples were exhaustively extracted with hexane, petroleum ether and ethanol. The solvent was evaporated at low temperature under reduced pressure in rotary evaporator to obtain crude extract. The yield percentage was calculated for petroleum ether extract and ethanol extract while hexane extract was tested for bioassays.

3. Bioassays

3.1 Anti-Fungal Assay: Anti-Fungal Assay was done by using agar tube dilution method (Choudhary *et al*., 1995). The test sample was prepared by dissolving 24 mg of crude extract and 12 mg of pure compound in 1ml sterile DMSO serving as stock solution. For preparing the media sabouraud dextrose agar was used for the growth of fungus. Media with acidic pH (pH 5.5-5.6) containing relatively high concentration of glucose or maltose (40%) was prepared by mixing (SDA) 32.5 gm 500 ml^{-1} distilled water. The contents were dissolved and dispensed as 4 ml volumes into screw capped tubes. They were autoclaved at 121 °C for 15 min. Tubes were allowed to cool to 50 °C and non-solidified SDA was loaded with 66.6 ul of compound pipetted from the stock solution. This gave the final concentration of 400 and 200 µg ml^{-1} of the crude extract (crude chloroform, hexane and methanolic extract) in the media. They were allowed to solidify at room temperature. Each tube was inoculated with 4mm diameter piece of inoculum removed from a seven-day-old culture of fungus. For non-mycelial growth, an agar surface streak was employed. Other media supplemented with DMSO and reference antifungal drugs was used as negative and positive control respectively. The tubes were incubated at 27-29 °C for 7-10 days. A relative humidity of 40-50% with an open pan of water was applied in the incubation room. Cultures were examined twice weekly during incubation. Growth in the media was determined by measuring linear growth (mm) and growth inhibition calculated with reference to the negative control.

3.2 Anti Leishmanial Assay

Promastigotes are the extracellular flagellated form of the parasite and they can be used for in vitro determination of leishmanicidic activity of natural compounds. Leishmanial promastigotes can be obtained from the infected animals or human in endemic areas. Leishmanial promastigotes were cultured in sterile 25 cm^2 tissue culture flask in tissue culture medium M-199 supplemented with 25 mM HEPES and 10% HIFBS (heat inactivated foetal bovine serum) at 25 ^{0}C. Parasites were centrifuged at 3000 rpm, diluted in minimum volume of PBS and were counted with the help of improved Neubauer chamber under a microscope. Parasites were diluted with the fresh medium to a final concentration of 2.0 x 10^6 parasites ml^{-1}. One mg of compound was dissolved in 50 µl of absolute MeOH or DMSO (Dimethyl sulfoxide) and the volume was made up to 1.0 ml with the culture medium. In a 96 well microtiter plate, 90 µl of the parasite culture (2.0x 10^6 parasites ml^{-1}) was placed and 10µl containing various concentrations of the experimental compound was added in the culture. Ten ul of PBS (phosphate buffered saline, pH 7.2 containing 0.5% DMSO) was added as negative control while amphotericin B, and pentamidine (to a final concentration of 1.0 mg ml^{-1}) were added separately as positive control. The plates were incubated at 25 ^{0}C in the dark for 3-5 days during which control organism multiplied 3-6 times. The culture was examined microscopically on an improved Neubauer chamber and ED_{50} value of compounds possessing anti-Leishmanial activity was calculated.

3.3 Brine Shrimp Lethality Assay (Cytotoxicity)

Bioactive compounds are often toxic to *Artemia salina* (Leach) shrimp larvae. The eggs of the brine shrimp *Artemia salina* when placed in artificial sea water, the eggs hatch within 48 hours, providing large number of larvae. This is a rapid, inexpensive, general bioassay, which has been developed for screening and monitoring of physiologically active natural products (Carballo *et al*., 2002), The eggs were stored at low temperature (4°C), they remain viable for years. The hatching tray (a rectangular dish (22×32 cm)) was half-filled with filtered brine solution and eggs of brine shrimp (50 mg) were sprinkled. They were incubated at 37 °C. Test sample (20 mg) was dissolved in 2 ml of respective solvent and from this solution transferred 5, 50 and 500 µl to vials. The final concentration was 10, 100 and 1000 µg ml^{-1}) respectively. The solvent was allowed to evaporate overnight. After 2-days of hatching and maturation as nauplii, 10 larvae/vials were placed using a Pasture pipette. The volume was made to 5ml with seawater. They were incubated at 25-27ºC for 24hrs under illumination. Solvent and reference cytotoxic drug serving as negative and positive controls were also prepared. Etoposide (LD_{50} = 7.465 µg ml^{-1}) was used as the standard reference cytotoxic drug. The survived brine shrimps were counted. The data was analyzed with Finney computer program to determine LD_{50} values with 95% confidence intervals.

3.4 Contact Toxicity Insecticidal Assay:

This test is used to assess the direct insecticidal actions of pure natural products or plant extracts. This method unambiguously demonstrates if a compound or extract is lethal to certain types of insects on contact [13]. Suitable quantities (1000, 500, 100, 50, 10 ppm) of test samples were dissolved in a volatile organic solvent and these solutions were then coated on the inner surface of 20 ml glass vials (two to five replicates for each concentration). Each glass vial was rotated by hand until the test solution was distributed on the vial inner wall and floor, and the solvent had mostly evaporated. Then each vial was placed in a fume hood for 10 minutes to ensure complete removal of the carrier solvent. When the solvent was completely evaporated, five test insects (or larvae) were placed carefully in each vial with sufficient food (i.e. the natural diet for that particular insect, such as leaves, grain, etc., or artificial diet which is different for different insects). The survival of the insects was assessed after 24-48 hours. Controls consist of test insects (or larvae) in vials, treated only with the carrier solvent. Survival of such controls should average over 95%. Data was analyzed with a Finney computer program (Probit analysis) to determine LC_{50} values and 95% confidence intervals.

3.5 Anti-bacterial Assay

Anti-bacterial assay was determined by using Agar well diffusion method [14]. Nutrient agar plates were swabbed with a 2-8 h broth culture of respective bacteria. Wells (6 mm diameter) were drugged in the media in each of these plates using a sterile metallic borer with centers at least 24 mm apart. Samples [1 ml (3 mg ml^{-1} of DMSO)] were then added in their respective wells using sterilized dropping pipettes. Other wells were supplemented with DMSO and reference antibacterial drug (Imipenum, 100 µg ml^{-1}) serving as negative and positive controls, respectively. The plates were immediately incubated at 37 ºC for 14- 19 h. Activity was determined by measuring the diameter of zones showing complete inhibition (mm). Growth inhibition was calculated with reference to positive control.

4. Results and Discussion

Recently, much attention has been directed toward biologically active compounds extracted from popular plant species. The crude Hexane extract was screened for their lethality bioassay, insect static activity, antibacterial activity, antifungal activity, and anti-Leishmanial studies but apium was found to have positive activity only against some strains of fungi. Antifungal activity of the subject plant was tested against *Trichphyton longifuss, Candida albicans, Aspergillus flavus, Microsporum canis, Fusarium solani* and *Candida glabrata.* Concentration of each sample was taken as 200 µg ml^{-1} of DMSO. Growth in the medium containing the extract was determined by measuring the linear growth in mm and % growth inhibition was calculated with reference to the negative control. The standard drug used was Micanazole 70 and Micanazole 98.4. The result indicated that the crude Hexane extract showed strong inhibitory activity against *Trichphyton longifuss* (80%) and *Microsporum canis* (80%).

Table 1: *In Vitro* Antifungal Bioassay of *Apium graveolens* Linn

Name of Fungus	Linear Growth (mm)		% Inhibition	Std. Drug MIC µg mL^{-1}
	Sample	Control		
Trichophyton longifusus	20	100	80	Miconazole
Candida albicans	100	100	0	Miconazole
Aspergillus flavus	100	100	0	Amphotericin B
Microsporum canis	20	100	80	Miconazole
Fusarium solani	100	100	0	Miconazole
Candida glabrata	100	100	0	Miconazole

Pathogenic fungi, dermatophytes, have the ability to invade keratinized tissues of animals and humans and cause a disease, dermatophytosis, which is the commonest human contagious fungal disease [15]. Trichophyton longifuss is responsible for a pathogenic characteristic, cutaneous mycosis which causes a severe type of acute inflammatory infection of the hair and follicle known as Favus. It results in permanent hair loss and sometime infects the nails and skin. Microsporum canis is an animal pathogen responsible for cutaneous mycosis. It is the most common cause of ring worm infection of hair and skin in dogs and cats. Human infection usually acquired by contact with infected animals, particularly cats.

Extractive value of different plant parts and celery of *Apium graveolens* Linn with ethanol and petroleum ether is reported in Table 2.

Table 2: Percent yield with solvent extraction of the aerial parts of *Apium graveolens* Linn

Apium graveolens Linn	Ethanol	Petroleum-ether
Flowers	2.5	0.7
Leaves	0.3	0.28
Market Celery	6.82	9.7
wild Celery	7.83	10.33

The percentage crude yield of wild seeds, flowers, leaves and cultivated seeds with ethanol was 7.83%, 2.5%, 0.38% and 6.82% respectively. Whereas the percentage extractive value with pet- ether for wild seeds, flowers, leaves and cultivated seeds were 10.3%, 0.7%, 0.28% and 9.72% respectively.

5. Conclusions

Results of the current work suggest that the assayed plant possess antifungal properties. The crude Hexane extract was found to have maximum antifungal effects against *Trichphyton longifuss* and *Microsporum canis.* Further research can be done to identify the active principles responsible for the antifungal activity of *Apium graveolens Linn.*

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