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Biochemical analysis and antimicrobial activity of the extracts of *Calotropis procera* and *Ocimum basilicum*

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Abstract
An experiment was conducted to determine the biochemical and antimicrobial attributes of crude plant extracts of *O. basilicum* and *C. procera*. 10% leaf and flower extracts were prepared in distilled water, acetone and methanol.
Crude extracts of both *C. procera* and *O. basilicum* plants showed total sugars 6.35 - 6.5, 4.37 - 4.8 mg/ml, reducing sugars 0.26 - 0.49, and 0.22 - 0.4 mg/ml, protein 1.2 - 1.31 and, 2.3 - 2.37 mg/ml, respectively. Similarly, ten free amino acids and four sugars were identified from visualized zones through thin layer chromatography (TLC). Both the plants extracts showed bioactivity against four pathogenic bacterial and three fungal species. Leaf extracts of *C. procera* showed significant bioactivity against *S. pneumoniae* and *E. coli* recording 20.0 and 17.0 mm zone of inhibition.
It is revealed that there may be correlation in between sugars, proteins and free amino acids with antimicrobial potential of *O. basilicum* and *C. procera* extracts.

Keywords: *Calotropis procera*, *Ocimum basilicum*, antimicrobial

Introduction
The antimicrobial is a compound used for the treatment of infection, which are caused by different type of microorganism such as bacteria, fungi, and protozoa. The word antimicrobial or antibiotics activity have been derived from Greek word (Anti = against, bios = life). The history of antimicrobial coincides with evolution of modern science. Its foundation was laid on the research work of Pasteure and Joubert, who discovered a special type of microorganism that stopped the growth of another microorganism (Kumar *et al*., 2010) [15]. Medicinal plants have played a very essential role in the development of human culture. Medicinal plants are main resources of traditional medicines and many of the modern medicines are produced indirectly from plants. (Saleh Hosseinzadeh 2015) [25]. Plants are capable to synthesize substances which have therapeutic tendencies (Geissman, 1963) [9] to treat various diseases. Many plants species are important source of drugs showing anti-cancer, antimicrobial, anti-inflammation, antioxidant and anti-diabetic properties. Plant extracts have been used as natural antimicrobials in food (Hsieh and Mau, 2001) [11].
Calotropis procera Linn is a wild growing plant of family *Asclepiadaecae*, which is known to possess different medicinal properties. The plant attains height of about 6m and widely distributed in tropical regions (Irvine, 1961) [12] abundantly found in Pakistan, India, Bangladesh, Burma and sub Himalayan region (Kritikar and Basu, 1999) [14]. Various parts of *C. procera* plants have been used as Indian traditional medicine to treat ulcers, tumours, leprosy, piles as well as in abdomen, spleen and liver diseases (Kritikar and Basu, 1999) [14]. The root extracts are commonly used in the treatment of dyspepsia (Kumar and Arya, 2006) [16], leaves, bark and root are used as a curative agent for jaundice by various tribes of central India. Leaves of *C. procera* in water showed potentials to reduce total viable count (Shittu, *et al*., 2004) [28]. The plant is also known for its toxic properties that include dermatitis, iridocyclites and acts like a poison and produces lethal effects (Vadlapudi and Chandrashekar, 2010) [32].
Ocimum basilicum (family; Lamiaceae) or basil originated from shores of Mediterranean Sea and Middle East and now growing in gardens worldwide (Grieve, 1967) [10]. The Lamiaceae plants are source of spices and flavours for various food products worldwide with effective drugs in folk medicine especially in Africa and Asia. The extracts showed strong antibacterial and antioxidant properties (Sacchetti *et al*., 2004; Jirovetz *et al*., 2003) [24, 13]. Basil oil has a wide application in perfumery, as well as in dental and oral products. Basil seeds are used in constipation, diuretic, Diarrhoea and mucous discharges (Farnsworth and Bunyapraphatsara, 1992) [8] while leaves used in dyspepsia (Nadkarni and Nadkarni, 1976) [22], treatment of skin diseases, acne, insect stings and snake bites (Martin and Ernst, 2004) [19]. In addition extracts of basil also showed antimicrobial responses against various microorganisms (Sappakul *et al*., 2003; Adiquzela and Colab, 2005) [26, 1]. Present study was designed to evaluate the biochemical and antimicrobial potential of extracts of *Calotropis procera* and *Ocimum basilicum* against pathogenic species which are not reported in earlier studies.

Material and Methods
Fresh leaves and flowers of *O. basilicum* and *C. procera* were collected during June - September 2014 from the campus of University of Sindh, Jamshoro, brought in lab and identified through taxonomist in Institute of Plant Sciences. The leaves and flowers were washed thoroughly 2-3 times with water and dried at room temperature for one week.

Isolation of plant extracts

2.0 gram dried leaves and flowers of *C. procera* and *O. basilicum* were grinded in few ml of water, 70% methanol and acetone, centrifuged at 6000 rpm and supernatant was collected in bottle. The process was repeated for twice, raised volume up to 20ml and stored at -40°C for further analysis.

Quantification of bio-chemicals

Total sugars, reducing sugars and total proteins were quantified from leaves and flower extracts of *C. procera* and *O. basilicum*. Total sugars was determined through following the method reported by Montgomery (1960) as 0.5ml extract was taken in test tube, 2.5ml of conc. sulphuric acid and 50µl of 80% phenol solution were added, mixed thoroughly and stand for 15 minutes at room temperature. Finally absorbance was read against blank on spectrophotometer at 485nm. Glucose was used as standard for interpretation of results. For reducing sugars (Miller 1959) [20], 2.0ml of plant extract mixed thoroughly with 2.0ml of Dintrosalicylic acid (DNS) in the test tube. The reaction mixture was heated in boiling water tub for 5 minutes, cooled at room temperature and the absorbance was read by spectrophotometer at 540nm against blank. Reducing sugar concentration was calculated from standard graph prepared by using different concentrations of glucose as standard reducing sugar. Total proteins were quantified through Lowry *et al*., (1951) [17] method. 0.5ml of plant extract was mixed with 2.5 ml of alkaline copper solution in the test tube, wait for 10 minutes at room temperature and finally 0.250µl Folin-Cicalteu (1:1v/v diluted with d. H_2O) was added. After 30 minutes, the absorbance was read at 750nm against blank on spectrophotometer. Bovine serum albumin was used as standard protein.

Identification of free amino acids and sugars

Free amino acids and sugars in 10% water extracts of leaves and flowers of *C. procera* and *O. basilicum* were checked and identified through thin layer chromatography (TLC) using silica gel G-60 (Fluka). 0.2ml extract samples and amino acids standards were applied on activated TLC plates and developed with butanol-acetic-water (4:1:5 v/v/v) while for sugars identification, 0.2ml sample extracts and sugars standards were applied on TLC plates and developed in butanol, acetic acid and water (5:1:4 v/v/v) as solvent for TLC. After drying the TLC plates, the separated zones of free amino acids were visualized with freshly prepared ninhydrin (0.2g in 100ml acetone) solution and dried in oven for 5 minutes at 80°C while separated zones of sugars were visualized with aniline spray (0.46g aniline and 0.85g phthalic acid dissolved in 50ml n-butanol). Individual visualized zones of test samples were identified and Rf values of standard amino acids, sugars and test samples were calculated by using the formula

$$\text{Rf value} = \frac{\text{Movement of solute}}{\text{Movement of solvent}}$$

Bioactivity of Extracts

For the purpose of bioactivity of extracts four identified bacterial strains (*Escherichia coli, Klebsiella aerogenes, Staphylococcus pneumoniae, Staphylococcus aureus*) and three fungal species i.e. *Aspergillus niger, Aspergillus fumigatus* and *Mucor geophillus* were collected from Diagnostic Lab ISRA University Hospital, Hyderabad.

For antibacterial activity, about 0.1ml of each respective activated bacterial species (*E. coli, K. aerogenes, S. pneumoniae* and *S. aureus*) was inoculated and spread on to sterilized LB medium. About 3.0 mm^3 diameter holes or wells were made in each seeded petri plate. About 10µl crude extract was added in separately labeled wells and incubated over night at 37°C. For negative control, well was filled with 10µl of 70% ethanol. The efficacy of each sample extract was observed after 24 hours of incubation. The zones of inhibitions produced by these extracts were measured.

Antifungal activity was tested against a common pathogenic fungal species *A. niger, A. fumigatus* and *M. geophillus* using the diffusion plate method. 0.5 ml of the fungal spore suspension was thoroughly mixed with 100 ml of melted Potato Dextrose agar (PDA) and poured into sterilized Petri dishes. As the agar was set, wells of 3 mm^3 diameter bore were prepared on each of the seeded plate. Each well was filled with 10µl sample extract and Petri dishes were incubated at 37ºC for 72 hours. All the culture plates were examined after 72 hours and the zone of inhibitions were measured.

Results and Discussion

Plants are capable to synthesize substances which have therapeutic properties. Many of these compounds have antimicrobial potential that either kill or inhibit pathogen growth. In present study, some of the biomolecules such as total sugars, reducing sugars and total proteins in 10% water extracts of *O. basilicum* and *C. procera* were analysed, free amino acids were identified and their correlation was evaluated with bioactivity of sample extracts against pathogenic four bacterial and three fungal species as presented in tables 1 – 5. It was observed that total carbohydrates were higher (6.5mg/ml) in 10% water extracts of flowers of *C. procera* but reducing carbohydrates and total proteins were lower (0.26mg/ml and 1.31mg/ml respectively) in same extract (Table 1). Similarly total protein contents were higher (2.37mg/ml) in 10% water extract of flowers of *O. basilicum* but the total carbohydrates were lower (4.8mg/ml) in all the extracts studied (Table 1). It was observed that pH of these extracts exhibited acidic in nature in the range of 4.01 to 5.48. The pH of all extracts of both plants *O. basilicum* and *C. procera* was observed as 5.48 and lower. Dahot and Soomro (1997 reported 5.88 pH of the *Cardia latifolia* fruit. This may be due to presence of different phytoconstituents in different plants. In present study, Leaves and flowers extracts of *C. procera* were found to be comparatively high in total sugars compared to *O. basilicum* extracts. Total carbohydrates were obtained as 6.5mg/ml in 10% water extract of flower of *C. procera*. Plants contain variable amount of sugar contents in its parts. Other researchers also obtained the sugar contents from plants. Dahot and Soomro (1997) [6, 7] isolated total sugar contents from the extract of *Chenopodium album* which are slightly higher than our results. However, Naqvi *et al* (2011) [23] obtained 10.06 mg/ml and 12.21 mg/ml total carbohydrates from 20% methanol extracts of *A. nilotica* stem and *R. communis* leaves which are almost similar to our results. The

total protein of *O. basilicum* leaves and flowers 2.30 to 2.37 which is lower than total protein of *Ficus elastic* leaves 2.68 and higher than the *Papaya* 1.60 and *Ficus bengolensis* leaves 1.68mg/ml (M. Umar Dahot, *et al* 1999) [6, 7].

Some sugars and free amino acid were also identified through TLC in 10% water extracts of *O. basilicum* and *C. procera* leaves and flowers. The Rf values of visualized zones of samples were matched with the Rf values of standard sugars and free amino acids. The flowers of *C. procera* were found to have higher values of amino acids as six zones were visualized. The Rf values of these zones were matched with Rf values of aspartic acid, lysine, arginine, leucine, methionine and phenylalanine. Leaves of *O. basilicum* also showed six visualized zones, Rf values of five were matched with Rf values of glutamic acid, lysine, hydroxyl proline, isoleucine, methionine while Rf value of one zone was not matched with any Rf value of standard amino acid (Table 2). In flowers of *O. basilicum* four zones were visualized with two were matched with alanine and tyrosine and two were unknown (Table 2). According to results in table 3, four visualized zones of samples were matched with Rf values of four sugars i.e. glucose, ribose, lactose and fructose in leaves of *C. procera* while two unknown zones were also visualized along with glucose, ribose and fructose in flowers of *C. procera*. Similarly four known and one unknown spots were visualized in *O. basilicum* leaves (Table 3). On the basis of identified amino acids (table 2) and sugars (table 3), it has been confirmed that flowers and leaves of both plants *C. procera* and *O. basilicum* contained peptides or protein and sugar containing antimicrobial substances. Barman and Rai (2006) [5] also reported the presence of good proportion essential amino acids in plants that can be compared with egg proteins. Bahar and Ren (2013) [4] reported the wide spectrum role of antimicrobial peptides against microorganisms.

Plants based antimicrobial peptides either inhibit the growth or kill the pathogen. Several researchers have isolated these compounds from plants that showed responses against microorganisms. In present experiment, 10% water, acetone and methanol extracts of leaves and flowers of *O. basilicum* and *C. procera* were used against four bacterial and three fungal species. According to results in tables 4 - 6, 10% water extracts *C. procera* leaves and flowers showed bioactivity against all tested bacterial and fungal species with 3.0 to 10.5 mm of zones of inhibitions. Similarly 10% acetone extracts of *C. procera* leaves showed significant response against *S. pneumoniae* with 20.0mm zone of inhibition. Similarly methanol and acetone extracts of both plants failed to show effectiveness against *M. geophilus* (Tables 5 & 6) and only water extracts of C. procera leaves and flowers were effective against this fungal species (Table 4).

All 10% solvent extracts showed antimicrobial activity but this response was lower than other researchers, this may be due to use to 10% extracts while other researchers used 20% or higher concentrations of extracts for antibacterial and antifungal activities.

Phytoconstituents studies strongly confirm that small proteins or peptides play a significant part in plants as their antimicrobial defense system (Terras *et al*., 1995) [30], which protects them from microbial invaders. Plants secreted peptides or proteins and sugars have their own specific defense mechanism against plant pathogens. Among these, active mechanism may be either creation of ion channels in membrane of microbes (Zhang and Lewis, 1997; Terras *et al*., 1993) [33, 29] or microbial proteins are adhered to polysaccharide receptors of the host (Sharon and Ofek, 1986) [27]. Furthermore, plants also secrete low molecular weight phytoalexins that protect them against the microbial invaders (Maher *et al*., 1994; Van-Etten *et al*., 1989) [18, 31]. Zhang and Lewis (1997) [33] reported that polypeptides and polysaccharides such as starch, fabatin and various lectins are effective inhibitors of pathogens. According to Avorn (1996) [3], fructose present in cranberry inhibited the adsorption of *E. coli* to epithelial cells of urinary tract.

Table 1: Quantification of total proteins, total and reducing sugars water extracts of leaves and flowers of *O. basilicum* and *C. procera*

Plant Extracts	PH	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Total protein (mg/ml)
O. basilicum (Leaf)	5.07	4.87	0.27	2.3
O. basilicum (Flower)	5.48	4.8	0.4	2.37
C. procera (Leaf)	5.17	6.35	0.49	2.12
C. procera (Flower)	4.01	6.5	0.26	1.31

Table 2: Identification of free amino acids in 10% water extracts of leaves and flowers of *O. basilicum* and *C. procera* through TLC

Plants Extracts	Standard Rf Value	Sample Rf Value	Identified amino acids
O. basilicum (flower)	-	0.15	Unknown
	-	0.23	Unknown
	0.304	0.30	Alanine
	0.400	0.41	Tyrosine
O. basilicum (Leaf)	-	0.14	Unknown
	0.076	0.08	Glutamic acid
	0.207	0.21	Lysine
	0.312	0.31	Hydroxy proline
	0.433	0.43	Isoleucine
	0.525	0.53	Methionine
C. procera (flower)	0.100	0.11	Aspartic acid
	0.207	0.22	Lysine

	0.320	0.32	Arginine
	0.466	0.45	Leucine
	0.525	0.52	Methionine
	0.628	0.62	Phenylalanine
C. procera (Leaf)	0.207	0.20	Lysine
	0.307	0.30	Alanine
	0.426	0.42	Glutamine
	0.525	0.52	Methionine
	-	0.86	Unknown

Table 3: Identification of carbohydrates in 10% water extracts of leaves and flowers of *O. basilicum* and *C. procera* through TLC

Plants Extracts	Standard Rf Value	Sample Rf Value	Identified Carbohydrates
O. basilicum (flower)	0.21	0.2	Maltose
	0.34	0.345	Glucose
	-	0.48	Unknown
O. basilicum (Leaf)	0.34	0.35	Glucose
	0.42	0.421	Ribose
	-	0.52	Unknown
	0.83	0.825	Lactose
	0.91	0.92	Fructose
C. procera (flower)	-	0.26	Unknown
	0.34	0.33	Glucose
	0.42	0.415	Ribose
	-	0.75	Unknown
	0.91	0.90	Fructose
C. procera (Leaf)	0.34	0.33	Glucose
	0.42	0.41	Ribose
	0.83	0.82	Lactose
	0.91	0.91	Fructose

Table 4: Antimicrobial activity of water extracts of leaves and flowers of *O. basilicum* and *C. procera* against some pathogenic bacterial and fungal species

Plant Extracts	Antibacterial activity (Zones of Inhibitions in mm)				Antifungal Activity (Zones of Inhibitions in mm)		
	K. aerogenes	*E. coli*	*S. pneumoniae*	*S. aureus*	*A. niger*	*A. fumigatus*	*M. geophilus*
O. basilicum (Leaf)	6.0	4.8	-ve	-ve	6.0	-ve	-ve
O. basilicum (Flower)	-ve	10	-ve	-ve	-ve	-ve	-ve
C. procera (Leaf)	5.5	6.0	8.0	7.5	4.0	10.5	5.5
C. procera (Flower)	-ve	4.0	5.5	3.5	8.0	3.0	4.0
Negative Control (d. H_2O)	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 5: Antimicrobial activity of methanol extracts of *O. basilicum* and *C. procera* against some pathogenic bacterial and fungal species

Plant Extracts	Antibacterial activity (Zones of Inhibitions in mm)				Antifungal Activity (Zones of Inhibitions in mm)		
	K. aerogenes	*E. coli*	*S. pneumoniae*	*S. aureus*	*A. niger*	*A. fumigatus*	*M. geophilus*
O. basilicum (Leaf)	4.5	-ve	6.0	6.5	-ve	-ve	-ve
O. basilicum (Flower)	14.0	-ve	-ve	7.0	-ve	6.0	-ve
C. procera (Leaf)	8.0	7.0	-ve	5.0	6.5	18.0	-ve
C. procera (Flower)	4.0	5.5	-ve	-ve	10.5	8.0	-ve
Negative Control (70% Methanol)	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 6: Antimicrobial activity of acetone extracts of *O. basilicum* and *C. procera* against some pathogenic bacterial and fungal species

Plant Extracts	Antibacterial activity (Zones of Inhibitions in mm)				Antifungal Activity (Zones of Inhibitions in mm)		
	K. aerogenes	*E. coli*	*S. pneumoniae*	*S. aureus*	*A. niger*	*A. fumigatus*	*M. geophilus*
O. basilicum (Leaf)	-ve	7.5	12.0	-ve	8.0	-ve	-ve
O. basilicum (Flower)	-ve	16.0	8.5	-ve	-ve	-ve	-ve
C. procera (Leaf)	-ve	17.0	20.0	6.0	5.5	8.0	-ve
C. procera (Flower)	-ve	6.0	14.5	11.5	-ve	4.5	-ve
Negative Control (70% Acetone)	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Conclusion

The results revealed during the preliminary investigation on bioactivity of leaves and flowers extracts of *O. basilicum* and *C. procera* against pathogenic bacterial and fungal species and their correlation with sugars, proteins and amino acids. Leaves and flower extracts of *C. procera* showed significant

bioactivity against all tested microorganisms as the highest zone of inhibition (20.0mm) was observed against *S. pneumoniae*. *C. procera* extracts also showed higher amount of sugar contents compared with *O. basilicum* extracts. Results of sugars, proteins and free amino acids of both plants extracts revealed that these biomolecules may have correlation with the bioactivity against tested pathogenic microorganisms. Furthermore plants may contain alkaloids, phenolic acids, flavonoids, etc. which should be investigated.

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