ANTIMICROBIAL AND LARVICIDAL POTENTIAL OF SWEET BASIL (*OCIMUM BASILICUM* L.) EXTRACTS AGAINST LYMPHATIC FILARIASIS VECTOR *CULEX QUINQUEFASCIATUS*

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ABSTRACT: This study was designed to evaluate the antimicrobial and insecticidal potential of the extracts of medicinal plant *Ocimum basilicum* (basil). The plant parts including roots, leaves and shoots were dried and grinded into fine powder. Ethanol and acetone were used for crude extraction and later on extracts were dissolved in methanol. The antimicrobial activity of the extracts was determined by disc diffusion and agar well diffusion assays. The toxicity of the extracts was determined through microwell cytotoxicity assay against *Artimia salina*. Finally, insecticidal potential of the extracts was determined by larvicidal assay against *Culex quinquefaciatus* mosquito. The methanolic extracts exhibited an impressive antimicrobial activity against various Gram-positive and Gram-negative bacterial test strains. A promising larvicidal activity against *Culex quinquefaciatus* larvae was observed with larval mortality up to 80-90% in 48 hours. LC_{50} value for leaf extracts after 24 hours of exposure was 0.4 ppm and LC_{90} of 6.3 ppm. In contrast, lower cytotoxicity (35%) was observed against brine shrimp (*Artemia salina*) larvae after 24 hours of exposure. The chemical screening by TLC and HPLC/UV showed that the methanolic extracts contain active molecules belonging to various plants metabolites including indols, alkaloids and flavonoids. The study revealed that the extracts of *Oscimum bacillicum* could be exploited commercially as antimicrobials and as insecticides.

Keywords: *Ocimum basilicum*, antimicrobial activity, larvicidal activity, chemical screening and cytotoxicity.

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INTRODUCTION

Medicinal plants are a significant source of therapeutically important secondary metabolites and produce variety of antimicrobial, insecticidal, insect repellant and herbicidal compounds. *Ocimum basilicum*, commonly known as sweet basil, belongs to the family Lamiaceae and is mostly found in tropical and subtropical areas of Asia, Africa, Central and South America cultivated as ornamental or field crops (Tada *et al.,* 1996; Reuveni *et al*., 2002).

The plant leaves are rich source of essential oil found to be useful for the treatment of common colds, muscular spasms and as first aid treatment for the snakebites and wasp stings (Tanrıkulu *et al.,* 2018). The plant has also been conventionally used for the treatment of constipation, cough, headaches, warts and kidney problems (Vlase *et al*., 2014). In addition to medicinal properties of the plant, it is well known for its fragrance therefore commonly used in wide variety of perfumes and cosmetics (Baritaux *et al.,* 1992; Tanrıkulu *et al.,* 2018). Additionally, sweet basil is used as food preservative and flavoring agent in ice creams, condiments, non-alcoholic beverages and other foods. *O. basilicum* leaves mixed with Portugal "Serra da Estrela" cheese act as preservative by providing antioxidant activity, reducing moisture content and preserving unsaturated fatty acids and proteins (Carocho *et al*., 2016). Therefore, *O. basilicum* is truly a rich source of useful phytochemicals owing to which it is often referred as "king of herbs" (Makri and Kintzios, 2008).

The chemical characterization of *O. basilicum* extracts showed the presence of tannins, flavonoids, saponins and volatile terpenes like camphor, tymol, linalool and pinenes (Lorenzi and Matos, 2002). The antibacterial properties of *O. basilicum* are associated with its major components; estragole and linalool (Elansary *et al*., 2016; Mith *et al*., 2016; Avetisyan *et al*., 2017; Hanif *et al*., 2017).

Insect transmitted diseases are a major source of morbidity and mortality around the globe (Dhanasekaran *et al*., 2010). Among them, mosquitoes are the most important vector of disease, causing millions of deaths each year (El-Bendary *et al*., 2010). The major methods to control these diseases are by breaking the transmission cycle through vector control strategies. In ancient times, people utilized tars, smokes, plant oils and other substances for repelling insects (Peterson and Coats,

2001), which has been widely replaced by synthetic insecticides in the modern era. However, insecticides of chemical origin are dangerous to health and exhibit many side effects (Mandal, 2011). Plant extracts being safe are utilized in many parts of the world to control mosquito population (Sukumar *et al*., 1991).

Culex quinquefasciatus Say, commonly known as southern house mosquito, is the most common mosquito type found in Pakistan (Ashfaq *et al*., 2014). It is vector of many fatal human diseases including lymphatic filariasis (LF), St. Louis encephalitis virus (SLEV), Western equine encephalitis virus and West Nile virus (Hill and Connelly, 2009). Since many populations of *C. quinquefasciatus* have developed resistance to the most commonly used synthetic insecticides (carbamate, pyrethroid and organophosphate), there is a strong need to test for effective and environmental friendly alternative insecticides.

In present study, *O. basilicum* extracts were used to determine the antibacterial activity against Gram-negative and Gram-positive bacteria. Furthermore, the extracts were evaluated for cytotoxicity against brine shrimp (*Artimia salina*) larvae. Finally, larvicidal potential was determined against *Culex quinquefasciatus* mosquito larvae.

MATERIALS AND METHODS

Sample collection: *Ocimum basilicum* plants of the age 16-20 weeks having roots, stems, leaves and flowers were obtained from a nursery at Faisalabad, Pakistan.

Extraction: Modified method of Hassan and Sajid (2015) was followed for the extraction of bioactive plant metabolites. The plants were dried on blotting paper in sunlight for about a week. Afterwards, the dried parts of the plant were separated into roots, stems, leaves and flowers and grinded in electric grinder into fine powder. All the samples were transferred into a flask and gradually ethanol was added into it and the flasks were placed on a linear shaker for 3-4 days. The samples were filtered through whatman filter paper and again the sample powder were mixed with acetone and was placed on a linear shaker for 3-4 days and filtered. The solvents having extracts were recycled on a rotary evaporator and the extracts were mixed in methanol (Hassan and Sajid, 2015).

O. basilicum plant extracts (n=8) were prepared from its roots, stem, leaves and flowers after initial extraction with ethanol including Niazbo root extracts in ethanol (ERE), Niazbo stem extracts in ethanol (ESE), Niazbo leaf extracts in ethanol (ELE), Niazbo flower extracts in ethanol (EFE) and acetone: Niazbo root extracts in acetone (ARE), Niazbo stem extracts in acetone (ASE), Niazbo leaf extracts in acetone (ALE) and Niazbo flower extracts in acetone (AFE).

Antimicrobial activity and cytotoxicity: For antimicrobial activity panel of five bacterial isolates including: *Klebsiella sp., Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were used. The antimicrobial activity was determined by agar well diffusion method and disc diffusion assay using Luria Bertani agar medium. For agar well diffusion assay, bacterial lawn was prepared for each type of isolate by inoculating the petri plates by using sterile cotton swabs dipped in standardized inoculum suspensions. Then with the help of a sterile cork-borer, 6mm agar wells were made and 40µl of extract was loaded into each well and the plates were incubated for 24 hours at 37^{o}C. In case of disc diffusion assay, filter paper discs of the size of 6mm were prepared and were impregnated with 40µl of extract. The discs were placed on the surface of agar medium inoculated with bacterial isolate and the plates were incubated for 24 hours at 37^{o}C. Zone of inhibition (mm) was noted to determine the antimicrobial potential of the plant extracts.

For microwell cytotoxicity assay, artificial seawater was prepared in a flask and shrimp eggs were added in the flask, which was left in dark for 24 hours. After hatching of the eggs, about 30-40 larvae were transferred to each well of a microtiter plate along with 200µl seawater and about 20µl of the extract. The plate was left in dark for 24 hours and larval mortality was calculated using the method described by Tanvir *et al.*, (2014).

Larvicidal assay against *Culex quinquefasciatus* mosquito: Larvicidal potential of the plant extracts was evaluated following WHO pesticide evaluation (WHOPES) guidelines (2005) for laboratory and field testing of mosquito larvicides. Briefly, 25 fourth instar *Culex quinquefasciatus* larvae were added to the glass beaker containing 50ml of *O. basilicum* plant extract. Each experiment was carried out in five batches. For each assay distilled water was used as negative control, while for positive control, commercially available insecticide Permethrin, was used. The mortality of the larvae was determined after 24, 48 and 72 hours of incubation at 28±2°C (WHO, 2005).

Concentration response assay: Based on the preliminary screening results, the extracts were subjected to concentration-response assay for larvicidal activity against *C. quinquifaciatous* mosquito following WHO pesticide evaluation guidelines (WHO, 2005). The compounds showing ≥50% mortality in 24 hours alone were further evaluated for the concentration response assay. Concentrations ranging from 0.02 to 20 ppm were prepared and evaluated for the concentration response assay. The number of dead larvae at 0.02, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 20 ppm were counted after 24 hours of exposure, and the percentage

mortality was calculated from the average of five replicates (WHO, 2005).

Chemical profiling of the extracts: The methanolic extracts were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). For thin layer chromatography the method of Kirchner (1967) was followed. Briefly, small spots of solution having the extracts were applied on the plate 1cm from the bottom marked with a line. The sample spotted on the plate was allowed to dry before the plate was transferred to chromatographic tank, which contained dichloromethane/methanol (90:10) solvent system. The process was checked as solvent rises up the plate using mobile phase, after the plate was removed and air dried. After drying the plate, the spots were viewed under UV at 254nm and 366nm and the plate was sprayed with the staining reagent, anisaldehyde/H_2SO_4 for color production by different components (Kirchner, 1967).

HPLC was performed according to method described by Noureen and colleagues (2016) for the detection of compounds at different retention times (t_R) and their relative concentrations. The crude extracts were examined on the high performance liquid chromatography system (Sykam GmbH) using 30cm long C18 column (phemomenex®, USA). For mobile phase, methanol was used and flow rate was set at 1ml/min. The extracts of *O. basilicum* were mixed in high performance liquid chromatography grade methanol for sample preparation and 25µl sample was injected with the help of a micro-syringe. Extracts were allowed to run for 15 minutes and detector was set at 254nm. The peaks were observed at different retention times (t_R) and the data was compared with standard UV absorption data of plant metabolites (Noureen *et al*., 2016).

Statistical analysis: The data was analyzed in Microsoft Excel 2013. LC_{50} and LC_{90} was calculated through probit regression analysis in GraphPad Prism version 7.0. Percentage mortality and standard deviation was calculated from the mean of five replicates.

RESULTS

Antimicrobial activity and cytotoxicity: In agar well diffusion assay, *O. basilicum* ethanolic flowers extract (EFE) gave maximum zone of inhibition of 11, 12 and 12mm against Gram-negative bacteria *K. pneumoniae, E. coli* and *P. aeruginosa,* respectively. However, ethanolic stem extracts (ESE) and leave extracts (ELE) gave higher zones of inhibition against *B. subtilis* (16mm) and *S. aureus* (14 mm). The acetone leaves extracts (ALE) showed maximum zone of inhibition of 13, 14 and 12mm against *P. aeruginosa, B. subtilis* and *S. aureus,* respectively (Table 1). Similarly, leaves extracts (ELE) showed broad spectrum of activity in disc diffusion assay, showing maximum activity against *K. pneumonia* (14mm), *P. aeruginosa* (14mm) and *B. subtilis* (14mm). However, stem extracts (ESE) showed maximum activity against *S. aureus* (24mm). Acetone leave extracts (ALE) of *O. basilicum* showed broad spectrum of activity against both Gram-positive and Gram-negative bacteria, giving maximum zone of inhibition against *S. aureus* (Table 1). It is noteworthy that no activity was observed for ERE and AFE in disc diffusion assay.

In cytotoxicity assay, all the extracts of *O. basilicum* ethanolic leaves extract (ELE), ethanolic flowers extract (EFE), acetone leaves extract (ALE) and acetone flowers extract (AFE) showed up to 7, 35, 17 and 29 percent larval mortality against brine shrimp (*Artemia salina*) larvae after 24 hours of exposure (Table 1).

Larvicidal activity against *Culex quinquifaciatous:* Six *O. basilicum* extracts were tested for larvicidal potential in terms of percentage mortality after exposure for 24, 48 and 72 hours. Ethanolic extracts of *O. basilicum* (ESE, ELE and EFE) showed negligible larval mortality after 24 and 48 hours of exposure (Fig-1). Only EFE gave 50% mortality after 72 hours of exposure. Contrastingly, acetone extracts (ARE, ASE and ALE) showed better results as compared to ethanolic extracts. ARE, ASE and ALE gave 30, 50, and 50 percent larval mortality after 24 hours of exposure, which was further improved to 60, 60 and 83 percent respectively after 48 hours of exposure. Highest larval mortality of 90 percent was recorded for leaf extract ALE of *O. basilicum* after 72 hours of exposure. LC_{50} value for ALE was 0.4 ppm and LC_{90} of 6.3 ppm after 24 hours of exposure (Fig-2).

Chemical profile of the extracts: Under short UV (254nm) *O. basilicum* root extracts (NR) showed 2 bands and band color was pink, stems (NS) showed 3 bands and band color was light red, leaves (NL) showed 5 bands and color of band was red and flowers (NF) showed 4 bands and band color was reddish. The red color in bands showed the presence of high amount of indols and amines (Fig-3).

The HPLC/UV analysis of extracts of *O. basilicum* showed the distribution and diversity of metabolites by observing the graph for voltage on y-axis and time on X-axis. The peaks generated by every extract were examined for different retention time (t_R) and the data was compared with standard UV absorption data of secondary metabolites. The HPLC chromatogram of roots extract of *Ocimum basilicum* exhibited peaks at retention time (t_R) of 2.16, 2.40, 2.57 and 2.82 min. The stems extract of plant showed peaks at retention time (t_R) of 2.14, 2.42, 2.86 and 3.74 min. Leaves extract of plant showed peaks at retention time (t_R) of 2.28, 2.39, 2.81, 3.50 and 3.72 min. In addition, flowers extract of plant showed peaks at retention time (t_R) of 2.34, 2.79, 3.45 and 3.73 min (Fig-4).

Table-1: Antimicrobial activity (zone of inhibition in mm) and cytotoxicity (percentage mortality after 24 hours of exposure) to *Ocimum basilicum* plant extracts.

		Agar well diffusion assay					Disc diffusion assay					% Mortality
	Methanolic plant extracts	***K. pneumoniae***	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***K. pneumoniae***	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***Artimia salina***
Ethanol extracts	Root extract	9	8	8	11	12	Nil	Nil	Nil	Nil	Nil	Nil
	Stem extract	8	9	7	16	12	12	7	12	11	24	Nil
	Leaves extract	10	8	12	14	14	14	8	14	14	18	7
	Flower extract	11	12	12	15	7	9	7	12	13	19	35
Acetone extracts	Root extract	10	8	7	12	7	12	7	20	12	12	Nil
	Stem extract	10	12	7	18	8	7	8	7	14	24	Nil
	Leaves extract	8	9	13	14	12	Nil	7	20	16	30	17
	Flower extract	8	9	11	13	8	Nil	Nil	Nil	Nil	Nil	29

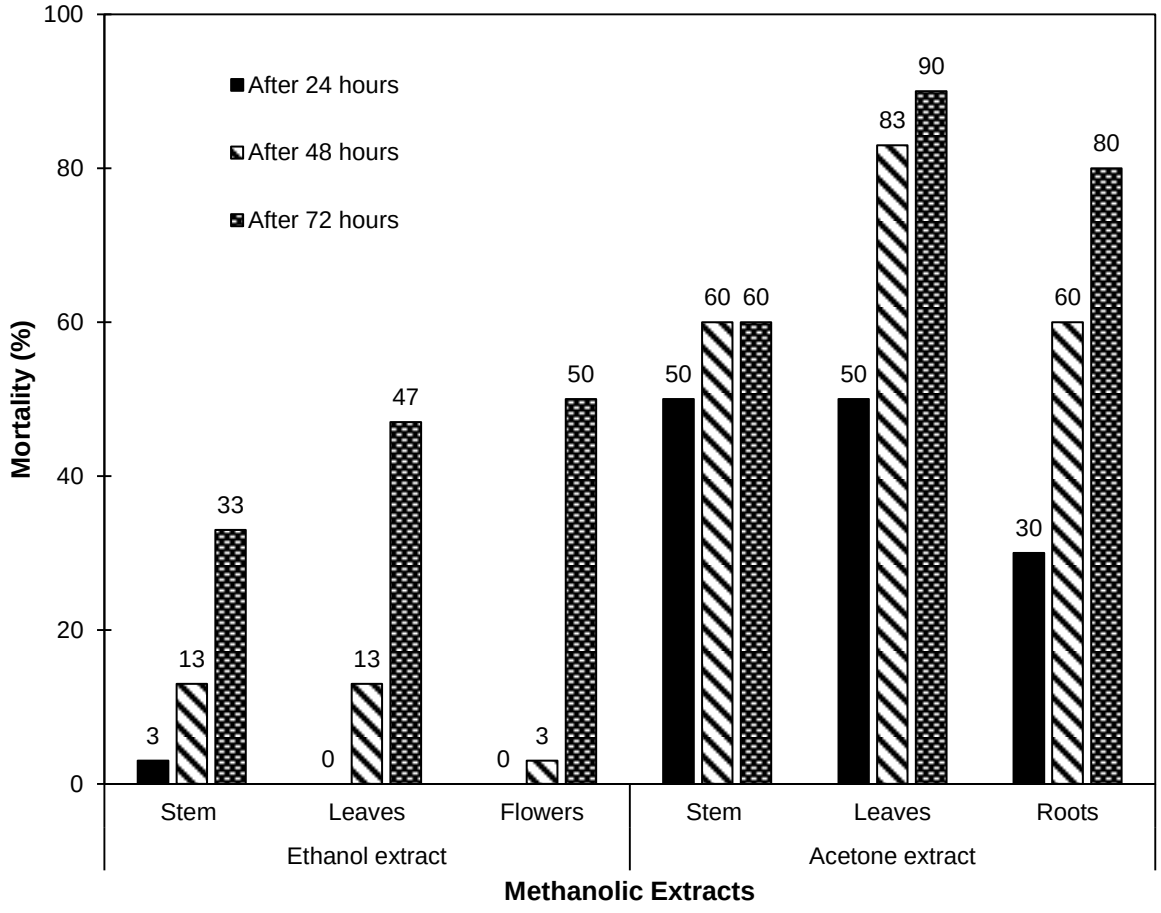

Figure-1: Larvicidal potential of *Ocimum basilicum* extracts against *Culex quinquefasciatus* after 24, 48 and 72 hours of exposure.

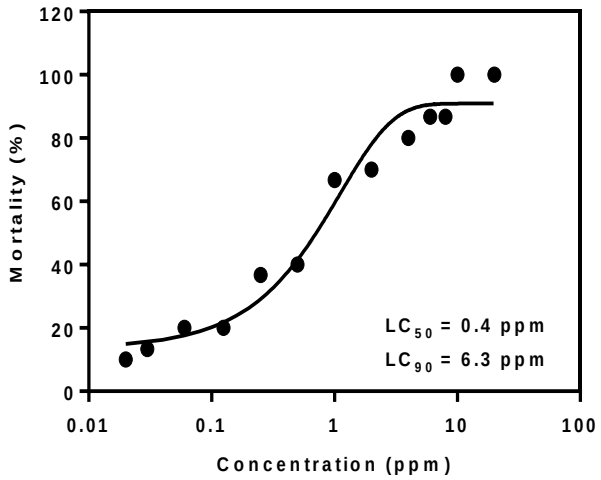

Figure-2: Concentration response curve showing larvicidal potential of the acetone leaves extract against *Culex quinquefasciatus* mosquito after 24 hours of exposure.

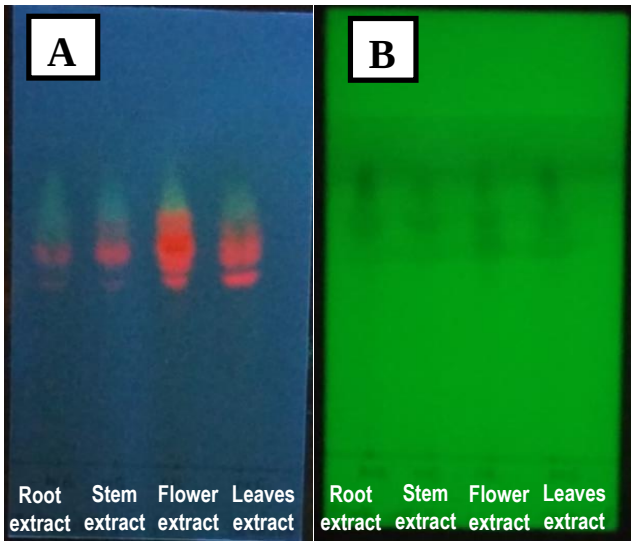

Figure-3: Thin layer chromatograph of *Ocimum basilicum* plant extracts (A): TLC plate under UV at 254 nm (B): TLC plate under UV at 366nm.

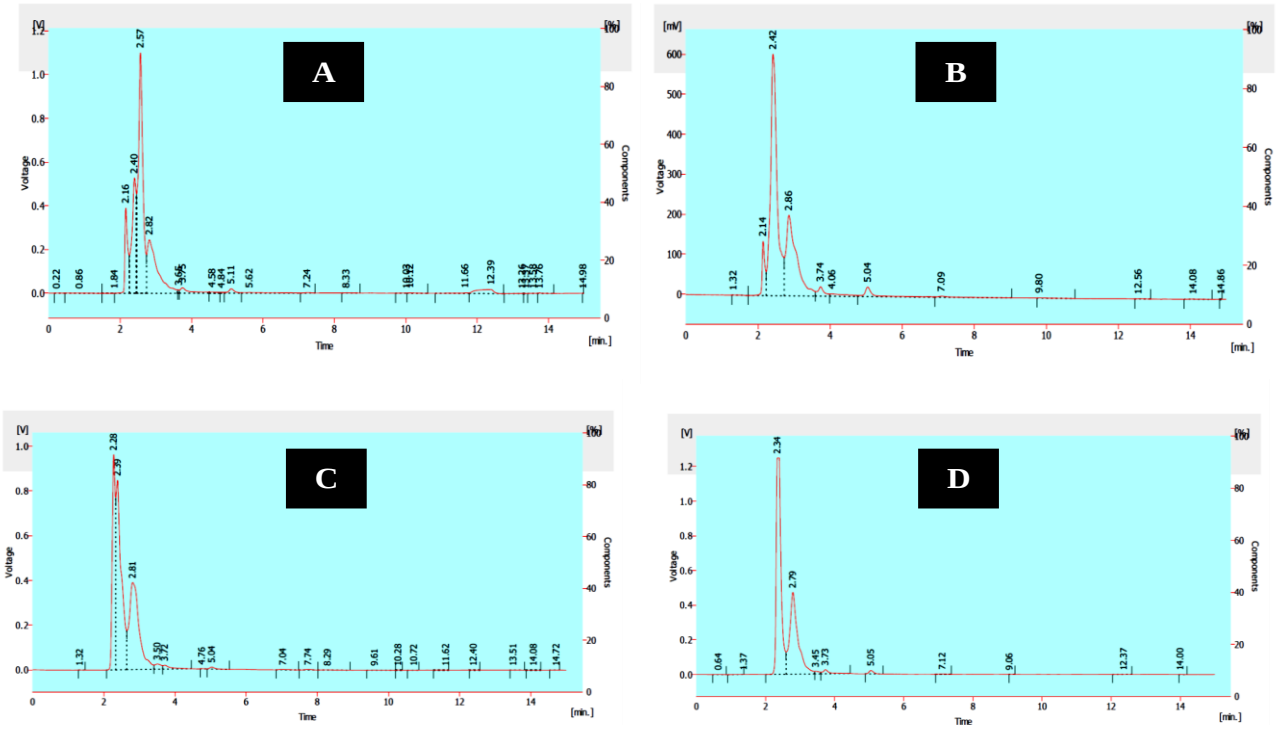

Figure-4: High performance liquid chromatography (HPLC) chromatograms (A) Chromatogram of root extract of *Ocimum basilicum* (B) Chromatogram of stem extract of *Ocimum basilicum* (C) Chromatogram of leaf extract of *Ocimum basilicum* (D) Chromatogram of flower extract of *Ocimum basilicum*.

DISCUSSION

Ocimum basilicum is an aromatic plant. Compounds obtained from this plant have been used in making medicines either in its original form or after some modifications (Nurzynska-Wierda *et al.*, 2012). *O. basilicum* exhibit broad spectrum of antiviral activity against medically important human viruses, including Herpes viruses, Adenoviruses, Hepatitis B virus, Coxsackievirus B1 and Enterovirus 71 (Chiang *et al.*, 2005). Various other studies suggest wide range of

pharmacological properties of sweet basil including curing cardiac disorders (Cohen, 2014), anticancer properties (Baliga *et al*., 2013, Nangia-Makker, 2013), antioxidant, antiulcerogenic, hypolipidemic, hypoglycemic, analgesic, anti-inflammatory and hepatoprotective, properties etc. in humans (Miraj and Kiani, 2016). The present study further evaluated antimicrobial and larvicidal potential of *O. basilicum* along with the chemical profiling of the active extracts.

A prominent antimicrobial activity response of the extracts of *O. basilicum* was found against gram-positive bacteria in comparison to gram-negative bacteria. Similarly, Moghaddam *et al*. (2011) reported higher activity of *O. basilicum* extracts against gram-positive bacteria as compared to gram-negative bacteria.

Liquid extract of *O. gratissimum* stopped the growth of Gram-positive and Gram-negativeve bacteria (Saha *et al*., 2013). While in present study, *O. basilicum* inhibited the growth of *Klebsiella sp., E. coli, P. aeruginosa, B. subtilis and S. aureus.*

O. basilicum was examined against 146 bacterial organisms. Ethanolic extracts showed the inhibition zones of 13-14mm against *E. coli* (Patil *et al.,* 2011). While in this study, the ethanolic and acetone extracts of *O. basilicum* showed maximum inhibition against *S. aureus* of 24, 18, 19mm in ethanolic and 12, 24, 30mm in acetone extracts. Runyoro *et al*. (2010) also described the activity of *O. basilicum* against *E. coli* and *S. aureus.* However, this study showed higher activity against *S. aureus* (max 30mm) while they were less active against *E. coli* (max 12mm).

Das *et al*., (2010) explained that acetone has efficiency to solubilize hydrophilic and lipophilic components of plant extracts. It is volatile and less damaging and very helpful in determination of bioactivity. Therefore, acetone could be used for efficient extraction of these types of bioactive plant metabolites instead of ethanol.

Furthermore, it is noteworthy that the maximum activity was shown by the leaf extract than the rest of the plant parts, which might be due to localization of these bioactive metabolites in the leaves of *O. basilicum.* Similarly, various other studies showed significant larvicidal potential of *O. basilicum* leaf essential oils against Culex larvae (Dris *et al*., 2017).

Various studies carried out previously reported effectiveness of *O. basilicum* extracts not only against different mosquito developmental stages (*i.e.* larvicidal, pupicidal and repellent activity) but also against different genera of mosquitoes. In this direction, a significant toxic effect of leaf extracts was reported against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* mosquitoes with an LC_{50} values of 14.01, 11.97 and 9.75 ppm, respectively (Govindarajan, 2013).

Additionally, current study reports high larvicidal activity of methanolic extract (ALE) against fourth instar *C. quinquefasciatus* larvae with LC_{50} value of 0.4 ppm and LC_{90} of 6.3 ppm after 24 hours of exposure. Recently, Dris and colleagues (2017) tested *O. basilicum* leaf extracts against fourth instar *C. pipiens* L. larvae and reported LC_{50} value of 73.45 ppm. Similarly, Govindarajan *et al*. (2013) determined toxicity of *O. basilicum* leaf essential oils and found significant toxic effect against late-third instar *C. tritaeniorhynchus* larvae with LC_{50} value of 14.01 ppm. Basil extracts were found to be effective against dengue/chikungunya vector *Aedes aegypti* mosquito. The essential oil of basil gave an LC_{50} value of 67.22 ppm against third instar larvae (Ramos *et al*., 2016). It is noteworthy that higher larvicidal potential of *O. basilicum* leaf extracts at much lower concentration has been noted in this study as compared to previous studies.

It is concluded that *O. basilicum* has good antimicrobial activity and the leaves extract of this plant exhibit very good larvicidal activity. People may grow this plant in their houses as a mosquito repellent. Considering the broad spectrum of activity of *O. basilicum* leaf extracts against mosquitoes, it could be used as newer, safer and effective alternative to chemical insecticides.

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