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*Full Length Article*

# Effect of Hexane and Ethanol Extracts of Ten Basil Genotypes on the Growth of Selected Bacterial Strains

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## Abstract

Bacterial infections are a nuisance to mankind from prehistoric times and still alive particularly in third world countries, where medicinal plants are extensively used as a treatment to pathogens since the known human history. In the current investigation, we used the crude extracts of an annual Holy Basil plant (*Ocimum basilicum* L. Family: Lamiaceae) enriched with a variety of aromatics, against the virulence of five pathogenic bacteria i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escheria coli, Proteus mirabilis* and *Klebsiella pneumoni.* Among the crude ethanolic and hexane extracts, the latter showed much higher antibacterial activity than the former. Phytochemical profiling confirmed the presence of certain organic compounds (terpenoids, flavonoids, phenolics, tannins and coumarines) in the crude basil extracts, bearing strong antibacterial properties. Antibacterial chemistry was studied through thin layer chromatography (TLC) analyses with selected Pakistani varieties of basil, and it was confirmed the presence of bioactive compounds like alkaloids and terpenoids (15% sulphuric acid and Dragedroff's reagent) in hexane extracts. The study sets further need for exploration of structure of these bioactive compounds with biological effect for pharmaceutical industry and future studies. © 2016 Friends Science Publishers

**Keywords:** *Ocimum basilicum*; Antibacterial; Thin layer chromatography (TLC); Pathogenic bacteria; Phytochemicals

## Introduction

New infectious diseases has been known since prehistoric times, which are inescapable however unforeseeable, well before the detection of pathogen even in modern age. Now day's extraordinary advances in development of countermeasures (diagnostics, therapeutics and vaccines), the ease of worldwide journey and increased global interdependence have added layers of complexity to containing these infectious diseases affecting not only the health but the economic stability of societies (Fauci and Morens, 2012). Pathogen borne diseases are common across the world and according to recent World Health Organization (WHO) statistics infectious diseases caused massive deaths of toddlers to early pubescent ages. Infectious diseases in non-industrialized countries may cause 45% in all and 63% of death in early childhood (Millar *et al*., 2007). According to WHO, up to 80% of the people depends on traditional system of medication and cure with medicinal plants across the globe (Arunkumar and Muthuselvam, 2009). For synthesis of complex chemical compounds and drug preparations, a good knowledge of the phytochemicals and source is desirable (Mojab *et al*., 2003; Parekh and Chanda, 2007, 2008). Phytochemical components such as tannins, carbohydrates, alkaloids, terpenoids, phenolics, steroids and flavonoids are responsible for various pharmacological disorders (Abbas *et al*., 2012; Zaman *et al*., 2012). These phytochemicals are synthesized as primary or secondary metabolites in tissues or cells of plants and secondary metabolites are taxonomically and chemically are diverse group of compounds with vague function mainly in pharmaceutics. Many of the phytochemicals are extensively used in agriculture as pesticides, insecticides and herbicides, in human medication and in therapies, veterinary science and many other branches of life sciences research (Vasu *et al*., 2009; Mansoor *et al*., 2011). The resistance of bacteria and fungi to antibiotics causes severe problem in agriculture and heavily impacted on economics of a country. Misuse and overuse of antimicrobial agents have exacerbated problems associated with development of resistance in microbes (Levy and Marshall, 2004). Increasing drug resistance presents of microbes is a major threat to public health because it may lessen the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality, and health care expenditure (Humeniuk *et al*., 2002). Natural products are important sources for preparation of biologically active drugs. There

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has been an increasing interest in the study of medicinal plants as source of natural products in different parts of the world (Gazzaneo *et al*., 2005).

Basil (*Ocimum basilicum* L.) having about 65 member in genus *Ocimum* belong to family *Lamiaceae*, is an annual herb and cosmopolitan in distribution and became the major cultivating as essential oil producing crop. Basil has a characteristic odor and sharp taste due to the presence of essential oils (Sajjadi, 2006), often referred as "king of herbs". *O. basilicum* is indigenous to South East Asia (i.e., Thailand, India, and Pakistan), but now cultivated worldwide to fulfill the needs of pharma industry. The medicinal value of basil can be determined by the bioactive constituents (alkaloids, phenolics, flavonoids, essential oils, tannins and saponins) (Krishnaiah *et al*., 2009) accountable for certain physiological action in the human body. Traditionally basil has been used for the treatment of respiratory and urinary tract problems. Basil is economically important due to the use of its essential oils being used in hygiene and cleaning products, perfumes, cosmetics, local anesthetic and antiseptics. Furthermore, basil essential oil has been tested in the control of plant pests, different human diseases, potent antioxidant and an antimicrobial (daCosta *et al*., 2015).

In order to contribute towards the solution for overcoming the side effects posed by excessive and massive usage of synthetic drugs against different ailments mainly resulting in anxiety and loss of natural immunity as well as economic burdens. The current study aims to evaluate antibacterial activity of the basil plants against five selected bacterial pathogenic strains. Phytochemical analysis for the screening of bioactive compounds and characterization of isolated extracts through chromatography fingerprinting was done for ten species of basil.

## Materials and Methods

### Collection of Plant Material

We obtained 10 basil species viz. Sweet Basil Japan, Italian Basil, Basil Siam Queen, Holy Basil, Hot wave Basil, Lime Basil, Garnet Basil, Green Basil, Basil Sindh, Thai Basil, from Pakistan Plant Genetic Research Institute (PGRI) Islamabad, Pakistan. The taxonomic position of the plants were identified and authenticated. The leaves of these species were separated and washed under clear running tab water and left for two weeks for shade dry. Dry leaves were then pulverized for grinding in electrical grinder and make fine powder of leaves.

### Preparation of Crude Extracts (Maceration)

Ethanol and hexane solvents were used for extracts preparation following maceration method. Dried leaf powder (30 g) was soaked in 150mL of solvents (hexane and ethanol) and kept on shaker for 48 h followed by filtration. The filtrates were then evaporated in rotary evaporator to get dried extract (2 g) which later kept at 4°C in refrigerator for further use.

### Collection of Bacterial Strains

Human pathogenic bacterial strains including one gram positive bacteria *Staphylococcus aureus* (*S. aureus*), and four gram negative bacteria *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escheria coli* (*E. coli*), *Proteus mirabilis* (*P. mirabilis*) and *Klebsiella pneumonia* (*K. pneumonia*) were kindly provided by Department of Microbiology, Quaid-i-Azam International Hospital Islamabad, Pakistan. These bacterial strains were maintained on nutrient agar at 4°C and sub cultured before testing.

*In vitro* antibacterial assay was performed following well diffusion method (Selvamohan and Sandhya, 2012). The minimum inhibitory concentration (MIC) was done by obtained by well method and plates were filled with nutrient agar. According to micro dilution method, we prepared gradual dilutions (20, 40, 60, 80 and 100 mg/mL in DMSO) of plant extracts according to the NCCLS protocols (NCCLS, 1992, 1997).Various concentrations of plant extracts prepared in hexane and ethanol (20, 40, 60 and 80 mg/mL) were tested against clinical bacterial isolates kept for 24 h maintained at 37°C, and each concentration zone gives different inhibition level. Phytochemical tests were carried out on hexane and ethanol solvents extract using standard procedures (Harborne, 1973; Trease and Evans, 1989). Column Chromatography separation was done involving the same principles as Thin Layer Chromatography (TLC), but can be applied to separate larger quantities than TLC. TLC of crude extracts of all genotypes were performed on silica coated TLC plate by using solvents system (hexane, chloroform and methanol).

## Results

### Effects of Ethanol and Hexane Extracts on Bacterial Growth

In the present study ten species of basil were selected (Egypt basil, Holly basil, Sweet basil, Hotwave basil, Green basil, Basil Sindh, Garnet basil, Italian basil, Siam queen basil, Basil lime) for their antibacterial efficacy using agar well diffusion method with crude extracts prepared in hexane and ethanol. Crude basil extracts prepared with ethanol and hexane solvents have varied percentage extracts (1.8–2.5 g), with the greatest concentration found in ethanol fractions. These basil extract were tested against five pathogenic bacteria (*S. aureus*, *P. aeruginosa*, *E. coli*, *P. mirabilis* and *K. pneumonia*) to analyze the highest antibacterial activity (Fig. 1a, b).

The zone of inhibition of hexane extract was in the range of (6–16 mm) among the tested strains (Fig. 1a). Hexane crude extracts of Green basil and basil Sindh gave

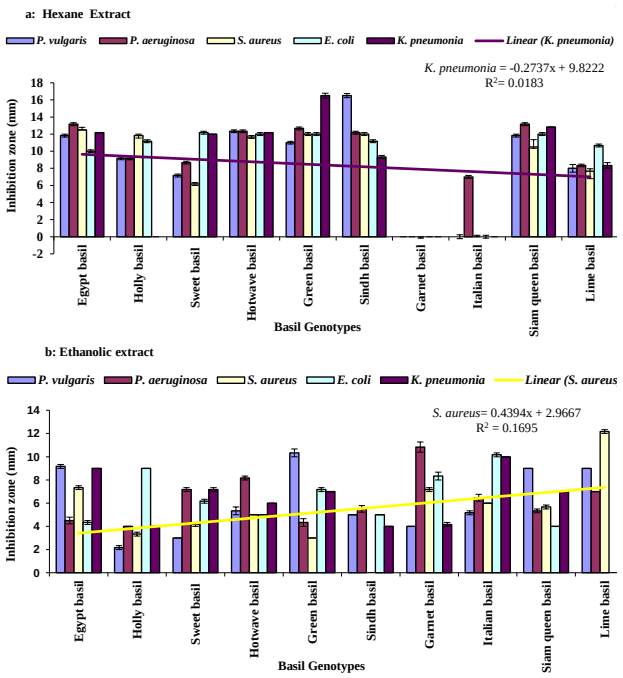


**Fig. 1:** The effect of hexane and ethanol extracts of ten basil genotypes on the growth of selected bacterial strains

maximum zone of inhibition (16 mm) against *P. mirabilis* and *K. pneumonia*, while Sweet basil showed minimum zone of inhibition (6 mm) against *S. aureus* (Fig. 1a). The ethanol extracts of basil Lime showed maximum activity against *S. aureus* with 12mm zone of inhibition (Fig.1b). The MIC results were significant ($p<0.05$) at 20 mg/mL for tested strains (Table 1).

Phytochemical screening through TLC confirmed the presence of alkaloids, terpenoids, flavonoids, phenols, tannins, and coumarines in the basil extracts which have been shown to possess antibacterial properties (Table 2). The growth of *P. aeruginosa* and *K. pneumonia*, *E. coli* and *P. vulgaris* were also significantly inhibited by basil hexane extracts. The hexane extract showed a stronger and broader spectrum of antibacterial activity than ethanolic extract (Fig.1b). Holly basil, Hotwave Basil, Egypt Basil and Siam Basil extracts were found to be effective against tested strains except Garnet basil. Italian basil was only effective against *S. aureus*. This might due to the nature and potentiality of biological active components (flavonoids, phenols, terpenoids etc.).

## Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by comparing the various concentrations of basil extracts with different inhibitory effects and the lowest concentration of extract showing inhibition were selected (Table 1). The ethanolic and hexane extracts gave a variable zone of inhibition of bacterial strains (Fig. 2a, b and 3a, d). Each crude extract concentration gave different zone of inhibition, and minimum zone of inhibition (2 mm) was given at 20 mg/mL, which we tested further, while maximum zone of inhibition (11 mm) at 80 mg/mL (Data not shown).

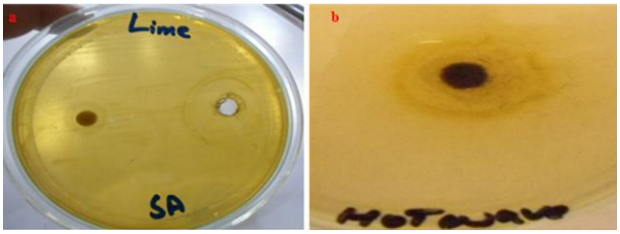


**Fig. 2:** Effect of ethanolic extracts on bacterial colonization measured by zone of inhibition (ZOI) at 20 mg/mL concentration. (a) Basil lime gave maximum (12mm) ZOI against *S. aureus*. (b) Hotwave basil gave 6mm ZOI against *K. pneumonia*

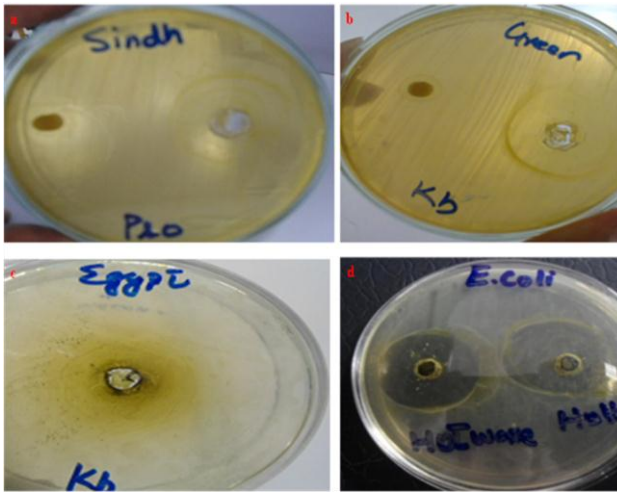


**Fig. 3:** Effect of hexane extracts on bacterial colonization measured by zone of inhibition (ZOI) at 20 mg/mL concentration.(a) Sindh basil gave ZOI against *P. vulgaris* (16 mm). (b) Green Basil gave 16mm ZOI against *K. pneumonia*.(c) Egypt basil gave 12mm ZOI against *K. pneumonia*. (d) Hotwave and holly basil gave 12mm ZOI against *E. coli*

**Table 1:** Minimum Inhibitory Concentration (MIC) Values at 20 mg/mL Concentration

| Basil genotypes | Bacterial strains (zone of inhibition) | | | | |
|---|---|---|---|---|---|
| | PRO | PA | SA | *E. coli* | KB |
| Egypt basil | 4 | 5 | 4 | 4 | 4 |
| Holly basil | 3 | 4 | 4.5 | 3 | 0 |
| Sweet basil | 2.9 | 3.1 | 0 | 4.3 | 5.6 |
| Hotwave basil | 4.2 | 6 | 4.1 | 5 | 4 |
| Green basil | 6 | 6.3 | 4 | 4 | 9 |
| Sindh basil | 9.5 | 4 | 4 | 4 | 3 |
| Garnet basil | 3 | 4 | 3 | 4 | 2 |
| Italian basil | 0 | 0 | 0 | 0 | 0 |
| Siam queen basil | 4 | 3 | 3 | 4 | 4 |
| Basil lime | 0 | 0 | 0 | 0 | 0 |

**Table 2:** Presence of different natural compounds in basil genotypes

| Compound class | Test plants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Egypt Basil | Holly Basil | Sweet Basil | Hotwave Basil | Green Basil | Sindh Basil | Garnet Basil | Italian Basil | Slam Basil | Lime Basil |
| Flavonoids | + | + | + | + | + | + | + | + | + | + |
| Couramins | + | + | + | + | + | + | + | + | + | + |
| Tannins | + | + | + | + | + | + | + | + | + | + |
| Phenols | + | + | + | + | + | + | + | + | + | + |
| Quinines | - | - | - | + | + | - | + | - | - | - |
| Saponins | - | - | - | - | - | - | - | - | - | - |
| Terpenes | + | + | + | + | + | + | + | + | + | + |
| Glycosides | - | - | - | - | - | - | - | - | - | - |
| Alkaloids | - | - | - | - | - | - | - | - | - | - |

The selected ethanolic extract concentration (20 mg/mL) of basil plants gave highest (12 mm) ZOI against *S. aureus*, while minimum (6 mm) ZOI was shown by Hotwave basil (Fig. 2a, b). At 20mg/mL concentration of hexane extracts of basil plants, the ZOI remained higher compared to ethanol extracts (Fig. 3a, d). Basil Sindh and Green basil showed maximum zone of inhibition (16 mm) against *P. aeruginosa* and *K. pneumonia*, respectively (Fig. 3a, b). Whereas, Egypt basil and Hotwave basil gave 12 mm ZOI against *K. pneumonia* and *E. coli*, respectively (Fig. 3c, d). All other basil genotypes were significantly effective against tested bacterial strains except garnet basil and Italian basil (Fig. 1a).

Results showed that the lowest concentration (20 mg/mL) of basil extracts inhibited the growth of tested bacterial strains except Italian basil and basil Lime (Table 1). Similarly at this concentration (20 mg/mL), Garnet basil showed minimum (2 mm) and maximum (9 mm) zone of inhibition, while all other basil genotypes gave different zones of inhibition between this ranges. Similar results for the MIC of cinnamon, clove, peppermint, nutmeg and lemon oils against various microbes (*S. aureus*, *Bacillus subtilis*, *B. cereus*, *E. coli*) were found to be in range of 1.25–6.25% (v/v) (Gupta *et al*., 2008).

In addition, basil genotypes were screened for the presence of phenols, terpenoids, flavonoids, coumarines, tannins, saponins, alkaloids and glycosides in hexane extracts (Table 2). Results indicate that basil genotypes showed positive results for phenols, terpenoids, flavonoids, coumarines, tannins, whereas alkaloids especially saponins and glycosides were not significant in hexane extracts (Table 2) might be due to the extraction solvent.

## Column Chromatography

The crude hexane extracts were fractionated by column chromatography, where the solvent passes through the column, the bioactive compounds move along the solvent and collected in glass tubes (Fig. 4a). Online thin layer chromatography (TLC) was done in order to know about the compounds in each elusion and 150 elusions were collected and dried. Crystals were also seen in some vials (Fig. 4b) which indicates the presence of some pure compounds which needs further investigations. Column elusions may contain these bioactive compounds. Elusions can be used for further

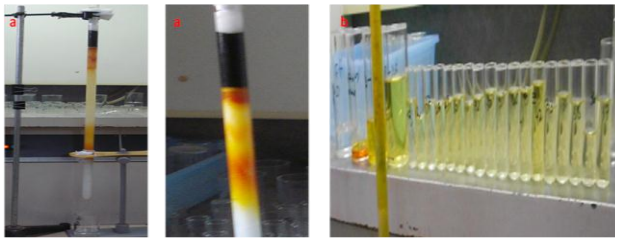

**Fig. 4:** Column Chromatographic analysis of crude extracts of basil genotypes. (a) Columns shoed bioactive compounds coming in the form of coloured bands. (b) Column elusions left to right in same pattern as in Fig. 1a

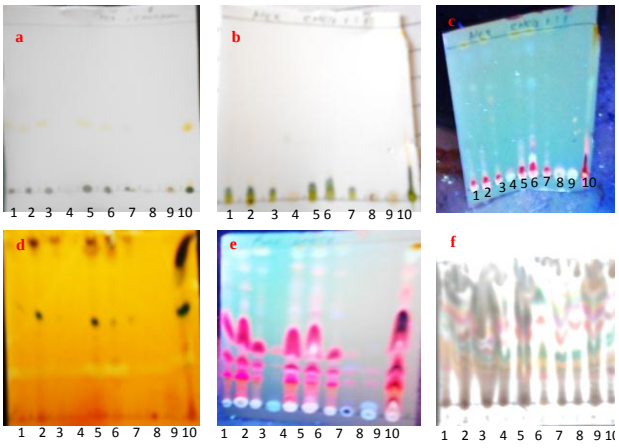

**Fig. 5:** Thin Layer chromatography (TLC) analysis in different solvents. (a) Movement of compounds in hexane solvent. (b) Movement of compounds in hexane: chloroform solvent (10:1). (c) Separation of compounds in hexane: choloroform (10:1) solvent under long wave (365 nm) UVR. (d) Movement of compounds in Dragondroff reagent for the detection of alkaloids. (e) Movement of extracts in chloroform under longwave (365 nm) UV for detection of terpenes (Purple bands), (f) the same plate rinsed in 15% $H_2SO_4$ for the detection of various compounds mentioned in Table 2

From Left to right: 1. Basil Sindh, 2. Green basil, 3. Holly basil, 4. Egypt basil, 5. Siam basil, 6. Sweet basil, 7. Italian basil, 8. Garnet basil, 9. Basil lime, 10. Hotwave basil. Differential movement of compounds according to their polarity in different solvents

identification and structure determination of major compounds, which are effective for drug development.

**Thin Layer Chromatography (TLC) Analysis**

The TLC of hexane and ethanol extracts showed slightly different banding pattern on TLC plates, and plates were visualized under short and long wave UVR (254 and 365 nm) (Fig. 5a, f). In pure hexane solvent, yellow colored bands move equally from crude extract of all basil genotypes on TLC plate (Fig. 5a). In hexane and chloroform solvent (10:1), more compounds move on plate (Fig. 5b), and under long wave UV light same plate gave maximum and obvious separation of compounds (Fig. 5c). Least amount of alkaloids were also observed indicated in orange colour (Fig. 5d), might due to less activity of solvent used for the screening of alkaloids.

## Discussion

The bioactivity of these natural compounds of basil could be enhanced in the presence of hexane because of its stronger extraction capacity, which squeezed greater amount of active constituents bearing antibacterial activity. All tested basil genotypes showed different antibacterial potential due to high degree of variation in the chemical composition of basil extracts. Goyal and Kaushik (2011) and Mann (2012), reported results against similar pathogenic bacterial strains following treatment of extracts of different plants. This indicated that plant extracts contain essential compounds that retard or inhibit bacterial growth, breakage of cell membrane and make it permeable causing leakage of ions and molecules (Kamba and Hassan, 2010).

It was assumed that the antibacterial activity of the basil extract may be due to presence of these compounds and it is suggested to explore the presence of further compounds in extracts may appeal the future studies. Previously, Bidarigh *et al*. (2012) reported the presence of antibacterial phytochemicals like flavonoids, phenols, terpenes and absence of alkaloids in hexane extracts of *Ocimum* and *Nerium oleander*, but the exact mechanism of antibacterial action of these compounds is still unclear. Most of studies revealed that, phenolic compounds interact with biomembranes of the cells. Terpenes possess antibacterial activity mainly affect cellular permeability and physiology furthermore, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. Plant phenolics constitute one of the major groups of compounds responsible for antioxidant behavior, as well as for antimicrobial effects (Marghitas *et al*., 2011). On the other hand flavonoids are also diverse group of natural compounds, are another important class of natural phenolics that possess a broad spectrum of biological activities, including free radical scavenging properties and antibacterial effects (Marghitas *et al*., 2011).

The presence of alkaloids in various extracts of *Ocimum* were also reported previously (Koche *et al*., 2012; Ojo *et al*., 2013; Janbaz *et al.*, 2014), but herein least amount of alkaloids were observed indicated in orange colour (Fig. 5d), might due to less activity of solvent used for the screening of alkaloids. Raja *et al*. (2012) confirmed the presence of terpenes in *Ocimum* species, similarly herein purple colour appeared on TLC plates when we applied 15% $H_2SO_4$, confirmed the presence of terpenes in basil extracts (Fig. 5e). TLC and phytochemical analyses confirmed the presence of terpenes, phenols, flavonoids, and coumarines (Fig. 5f) in our study and are in conformity with the previous reports (Mahalingam *et al.*, 2011; Koche *et al*., 2012; Prasad *et al.*, 2012; Ojo *et al*., 2013). Among the two solvents, hexane crude extracts showed a broad spectrum of antibacterial activity. Antibacterial assay revealed that crude extract of basil exhibited a broad spectrum of antibacterial activity.

## Conclusion

The hexane basil extracts, contain flavonoids, terpenes, tannins, phenols and coumarines have been shown to possess maximum antibacterial properties. It is suggested that the basil species would have a potential to combat different infectious diseases caused by the bacterial strains enlisted in present manuscript. Hence basil can be seen as a potential source of drugs for sustainable future.

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