

## Comparative study for antibacterial potential of *in vitro* and *in vivo* grown *Ocimum basilicum* L. plant extracts

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**Abstract:** The antimicrobial activities of *in vitro* grown callus extract and *in vivo* grown *Ocimum basilicum* L. plant leaves extracts were studied and compared. Effect of extraction solvent was also assessed. These extracts were tested *in vitro* against eight bacterial strains following disc diffusion method. The results indicated that *in vitro* grown callus extracts of *O. basilicum* exhibited higher antimicrobial activity against tested Gram positive microorganisms as compared to *in vivo* grown plant material extract. These findings indicate towards potential use of biotechnology for natural therapeutic agent production.

**Keywords:** *Ocimum basilicum*, antimicrobial activity, tissue culture, medicinal plant, disc diffusion method.

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### INTRODUCTION

Plants have been used for centuries as remedies for human diseases. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world<sup>1,2</sup>. The World Health Organization (WHO) estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies for 80% of the world's population. WHO advocates that countries should interact with traditional medicine with a view to identify and exploit aspects that can provide safe and effective remedies for ailments of both microbial as well as non microbial conditions<sup>3</sup>. Herbs and plants used by traditional medicine practitioners contain a wide range of substances that can be used to treat chronic as well as infectious diseases. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action in the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds<sup>4</sup>.

In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have been developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases<sup>5,6</sup>. In order to find new therapeutic agents, plants having antimicrobial activity have attracted the attention of scientific community<sup>7-9</sup>.

*Ocimum basilicum* L. commonly called as Sweet Basil belongs to family Lamiaceae is native plant of Indo-Malayan region. It is called the "king of herbs" which contains plenty of phytochemicals with significant nutritional as well as antioxidant capabilities and health benefits.<sup>10</sup> Sweet basil is cultivated for production of essential oils, dry leaves as a culinary herb, condiment/spice or as an ornamental plant. It is used as an ingredient in

various dishes and food preparations, especially in the Mediterranean cuisine<sup>11</sup>.

Sweet Basil has shown unique health protecting effects due to its important flavonoids and volatile oils. The unique array of active constituents called flavonoids found in basil provides protection at cellular level. Orientin and vicenin are two water-soluble flavonoids that have been of particular interest in basil<sup>12</sup>.

Essential oil of basil, obtained from its leaves, has demonstrated the ability to inhibit several species of pathogenic bacteria that have become resistant to commonly used antibiotic drugs<sup>13</sup>. Due to its antimicrobial, insecticidal activity and very pleasant aroma, basil essential oil is widely used in the food, pharmaceutical, cosmetic, and aromatherapy industries<sup>14</sup>. In addition, now-a-days public prefers natural food additives hence naturally derived antimicrobial agents from basil have become more important in antimicrobial packaging as they present a perceived lower risk to consumers<sup>15</sup>.

Aromatic leaves and flowering parts of *O. basilicum* are traditionally used as stimulant and tonic agents used in folk remedies to treat various ailments such as poor digestion, stomach-ache, feverish illnesses, nausea, abdominal cramps, gastro-enteritis, migraine, insomnia, depression, gonorrhoea, dysentery, and chronic diarrhoea exhaustion<sup>16</sup>. Externally, they have been applied for the treatment of acne, loss of smell, insect stings, snake bites, and skin infections<sup>17</sup>.

A plenty of work has been done on sweet basil regarding its anti microbial properties by using different chemical extracts such as chloroform, methanol, acetone and n-hexane extracts<sup>18,19</sup>. However, *in vitro* grown *Ocimum basilicum* callus extract has not been reported in terms of antimicrobial activity.

The purpose of this study was to evaluate the potential antimicrobial activities of *in vitro* grown callus and *in vivo* grown *O. basilicum* plants in two different concentrations of methanol.

## MATERIALS AND METHODS

### Source and preparation of explant

Sweet basil (*O. basilicum* L.) seeds were purchased from the local market, germinated and grown in the garden of PCSIR Laboratories Complex, Ferozpur Road, Lahore for *in vivo* studies whereas leaves of these plants after sterilization were used as explant for *in vitro* studies. The explants were washed thoroughly under running tap water for 10 min, rinsed with 95% ethanol, treated with 10% commercial bleach (Kilite) for 8 min. and were surface sterilized for 4 min with 0.1% HgCl<sub>2</sub> containing 2-4 drops of tween-20 to ensure contamination free explants. Thereafter, these explants were rinsed thrice with autoclaved distilled water inside the laminar air flow cabinet.

### Medium composition and callogenesis of *O. basilicum*

The surface sterilized leaves were cut in to 0.5-1cm small pieces and inoculated on full strength MS medium<sup>20</sup> supplemented with different dosages of 6-Benzyl amino-purine (BA) in combination with 1.07μM Nephthalene acetic acid (NAA). BA was supplied from 0.44 μM to 8.88μM. pH of the medium was adjusted to 5.8 with the addition of NaOH / HCl as required prior to the addition of phytigel (0.2%). After dispensing in the culture tubes it was autoclaved for 20 minutes. at 121°C under a pressure of 15psi. After inoculation of surface sterilized leaf explants these cultures were kept for 21 days in a growth chamber for callogenesis. The growth chamber was maintained at 25±2°C under 16h photoperiod and 48μmol m<sup>-2</sup>s<sup>-1</sup> light intensity. Four replicates per treatment were used.

### Preparation of plant extracts

To prepare plant extracts absolute methanol (99%) and 70% aqueous methanol (70mL methanol:30mL distilled water) were used as solvent. Four kinds of plant extracts were prepared which includes *in vitro* grown callus extract (21 days old callus) and *in vivo* grown *O. basilicum* L. plants leaves extract both with absolute methanol and 70%aqueous methanol separately.

Took 10g each of *in vitro* or *in vivo* grown plant material, macerated in 30 ml of absolute or 70% of methanol individually and vigorously stirred with a

sterile glass rod separately and kept overnight. Extracts were occasionally shaken during 24h and then filtered through Whatman No.1 filter paper<sup>21</sup>discarding the plant material. Dirty green colored filtrate of *in vitro* grown calli and green colored filtrate of *in vivo* grown plant leaves were evaporated to dryness on a water bath at 100 °C. The dried extracts were sterilized by placing them under UV light for 24 hours. Each of the alcoholic extracts was reconstituted by adding 2mL of 10% aqueous dimethylsulfoxide (DMSO) with Tween-80 (0.5% v/v) which was sterilized by filtration through a 0.45 μm membrane filter.

### Test organisms

*In vitro* antimicrobial studies were carried out on eight bacterial strains (*Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae*, *Salmonella paratyphi* , *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter* specie) some of which were obtained from PCSIR laboratories complex, Lahore and other from pathological laboratory of a local hospital. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

### Antibacterial Assay

Paper disc diffusion method was applied to test the antimicrobial activity of the extracts. Discs of Whatman No.1 filter paper having a diameter of 6 mm were oven dried at 65°C overnight wrapped in tinfoil for sterilization.

Normal strength nutrient agar medium(OXOID, England) was prepared and autoclaved at 121°C and 15psi for 15 min. for culture growth through out the study for determination of antibacterial activity. For antibacterial assay 24h old bacterial cultures at 37°C were used. Cultures were diluted 10<sup>-1</sup> in sterile ringer solution<sup>22</sup> containing approximately 10<sup>6</sup>CFU/mL in each case. Twenty five micro-liters of these suspensions were inoculated over plates containing sterile nutrient agar medium using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates.

Filter paper discs each impregnated with 30μL of each plant extract were placed on pre-inoculated culture media under aseptic conditions separately and incubated at 37°C for 24h. The zone of inhibition in each case was measured as the diameter (in millimeters) of the clear zone around the discs. All experiments were performed in duplicate. Penicillin, co-trimoxazole and Streptomycin were used as positive controls. Inhibitory effect of positive controls was tested for

all microorganisms used in this study under the incubation conditions as mentioned above. The working solution of control antibiotics were prepared in appropriate amounts (0.01g/10mL) then 25µL of each antibiotic solution was dropped on paper discs and 10% aqueous solution of dimethylsulfoxide (DMSO) was used as negative control during this study.

### RESULTS AND DISCUSSION

Effect of different concentrations of BA (0.44-8.88µM) in combination with NAA (1.07 µM) on callus induction in leaf explants of *O. basilicum* L. is shown in table 1, observations were taken after one week. Explants responded to all concentrations of BA along with NAA producing calli having different attributes.

**Table 1:** Effect of NAA in combination with BA on callogenesis in *O. basilicum* leaf explants.

No.	Medium composition (µM)	Callus initiation (%)	Callus growth	Morpho-genetic potential	Remarks
1	MS+ 1.07 NAA +0.44 BA	40	+++	Root initiation	Callus Compact green
2	MS+ 1.07 NAA +0.88 BA	38	+	Nil	Callus Light green loose
3	MS+ 1.07 NAA +2.22 BA	70	+++	Nil	Callus somewhat yellowish vitrification
4	MS+ 1.07 NAA +4.44 BA	50	++	Root initiation	Callus Compact whitish green
5	MS+ 1.07 NAA +8.88 BA	100	++++	Nil	Callus Compact granular light green with powdery upper surface

+ = Poor  
 ++ = Good  
 +++ = Very Good  
 ++++ = Excellent

It was either compact, granular or powdery in texture where as its color varied from whitish green, light green, yellowish green or green. Superior sweet basil calli were produced on MS medium supplemented with BA (8.88µM) along with NAA (1.07µM) in terms of callus induction (100% explants). Whereas lesser quantity of BA (0.88µM) in the presence of 1.07µM NAA was found to be least effective for callus induction only 37.5% cultures showed callus induction as shown in table-1. These findings are in accordance to Dode et.al.<sup>23</sup> who reported that low auxin concentration (1.07µM NAA) combined with different levels of cytokinin (BA) was effective for callus induction.

The antimicrobial activities of *O. basilicum* in both *in vitro* callus and *in vivo* leaf extracts (absolute methanol or 70% aqueous methanol) against eight microorganisms were examined in the present study, the results are shown in table 2. It was observed that both extracts of *in vitro* grown callus were effective against two microorganisms *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* whereas *in vivo* grown leaf extracts were effective against only *Bacillus subtilis* ATCC6633 as shown in table 2.

**Table 2:** Assessment of antimicrobial activity in four different extracts of *O. basilicum* against eight microorganisms.

Micro-organisms	Zone of inhibition (mm)							
	MCE	ACE	MLE	ALE	DMSO	Streptomycin 25ug/disc	Penicillin 25ug/disc	Cotrimoxazol 25ug/disc
<i>E. coli</i> ATCC 25922	-	-	-	-	-	21	-	-
<i>B. subtilis</i> ATCC 6633	12.5	11.5	9.0	8.5	-	28	16.5	-
<i>K. pneumoniae</i> HI	-	-	-	-	-	18.5	-	-
<i>S. paratyphi</i> HI	-	-	-	-	-	14	-	-
<i>P. aeruginosa</i> HI	-	-	-	-	-	22	-	-
<i>S. aureus</i> HI	8.0	7.5	-	-	-	19	26	-
<i>E. coli</i> HI	-	-	-	-	-	24	-	-
<i>Enterobacter</i> HI	-	-	-	-	-	22	-	-

Absolute Methanol callus Extract = MCE  
 70% aqueous Methanol callus extract = ACE  
 Absolute Methanol Leaves extract = MLE  
 70% aqueous Methanol Leaves extract = ALE  
 Hospital isolated pathogen = HI  
 No inhibition zone (resistant) = (-)

All other bacterial strains were resistant to these extracts. These results depicted that *in vitro* callus extracts showed better antimicrobial properties as compared to *in vivo* grown leaves extract.

Effect of various extraction solvents used for *O. basilicum* L. on their *in vitro* antimicrobial properties were already studied<sup>18-19</sup> and reported that methanol extract of *Ocimum basilicum* L. was effective against six bacterial strains; namely *P. aeruginosa*, *Shigella sp.*, *L.monocytogenes*, *S. aureus* and two different strains of *E. coli*. It is also investigated *in vitro* antimicrobial properties and their results indicated that hexane was more effective in a wider spectrum as compared to methanol. In our study only two bacterial strains *S. aureus* and *Bacillus subtilis* showed inhibition against four tested methanolic extracts. These findings were contradictory previous studies, which has reported that methanol extracts were effective against *Pseudomonas aeruginosa* which is contradictory to our findings (Table 2). The present results showed that *Pseudomonas aeruginosa* was resistant to both *in vitro* and *in vivo* *Ocimum basilicum* extracts. In another study the extract of *O. basilicum* had antimicrobial properties against *E. coli*, *Salmonella paratyphi* and *Shigella dysenteriae*. These findings were not in corroboration with present results where *Salmonella paratyphi* and both strains of *E. coli* were resistant to tested *Ocimum basilicum* extracts<sup>20</sup>.

Streptomycin was effective against all tested microorganisms both gram positive and gram negative and the range of zone of inhibition was 14mm to 28mm whereas penicillin was effective against only Gram positive microorganisms *Bacillus subtilis* and *Staphylococcus aureus* and the range of inhibition zone was 16.5-26mm respectively. The results are shown in table 2. All tested microorganisms were resistant to Co-trimoxazole.

The results indicated that *Bacillus subtilis* and *Staphylococcus aureus* that resistant to standard antibiotic co-trimoxazole were sensitive to *in vivo* grown callus extract of *Ocimum basilicum* L. These findings are in accordance to Kaya *et. al.*<sup>19</sup> who reported that *Shigella sp.*, *P. aeruginosa*, *Escherichia coli* ATCC25922, *Escherichia coli* were resistant to a variety of standard antibiotics tested but methanol extract of *Ocimum* had antimicrobial activities on these microorganisms.

*O. basilicum* has an inhibitory activity on Gram positive bacteria which is as effective as antibiotics whereas the extracts of *in vitro* grown callus have larger potential of antimicrobial activity as

compared to *in vivo* grown leaf extracts of *O. basilicum*. High antibacterial activity of *in vitro* callus extracts may be due to culture conditions such as plant growth regulators provided in the culture medium for *in vitro* tissue growth. Effect of phytohormones for production of secondary metabolites has been reported<sup>26</sup> which support present results. Several bioactive compounds have been found to be accumulating in cultured cells at a higher level than those found in intact plants through optimization of culture conditions for example, ginsenosides by *Panax ginseng* and shikonin by *Lithospermom erythrorhizon*<sup>26</sup> were accumulated in much higher levels in cultured cell than in intact plants. It might be the reason for higher inhibitory capacity of callus extract than the *in vivo* grown plant extracts. It could be conferred from the findings of present study that use of *in vitro* grown basil material could be a better substitute of intact plant due to the presence of increased bioactive compounds effective against some microbes. These finding also indicate the potential use of Biotechnology for natural bioactive material production. It would be possible to produce new effective antibiotics from *Ocimum basilicum* using appropriate procedure/technology.

## REFERENCES

1. Ahmad I and Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J. Ethnopharmacol.*, 2001; 74: 113-123.
2. Saxena VK and Sharma RN. Antimicrobial activity of the essential oil of *Lantana aculeata*. *Fitoterapia*, 1999; 70: 67-70.
3. World Health Organization. Summary of WHO guidelines for the assessment of herbal medicines. *Herbal Gram*, 1993; 28; 13-14.
4. Edeoga HO, Okwu DE and Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *African J. Biotech.*, 2005; 4: 685-688.
5. Davis J. Inactivation of antibiotics and the dissemination of resistant genes. *Science*, 1994; 264, 375 -382.
6. Service RF. Antibiotics that resist resistance. *Science*, 1995; 270: 724-727.
7. Kalembe D and Kunicka A. Antimicrobial and antifungal properties of essential oils. *Current Med. Chem.*, 2003; 10: 813-829.
8. Fett-Neto AG, Melason SJ, Sakata K and DiCosmo F. Improved Growth and texol yield in developing calli of *Texus cuspidate* by medium composition modification. *Biotechnology*, 1993; 11: 731-734.
9. Juliani HR and Simon JE. In: Antioxidant activity of basil: A Trends in new crops and new uses, Fourth Edition, Editors: Janick J& Whipkey, VA: ASHS Press, Alexandria. 2002; pp 575-579.
10. Yayasinghe C, Gotoh N, Aoki T and Wada S. Phenolics composition and antioxidant activity of sweet basil (*Ocimum basilicum* L.). 2003; 51: 4442-4449.

11. Zheljzkov VD, Callahan AN, and Cantrell CL. Yield and Oil Composition of Thirty-Eight Basil (*Ocimum basilicum* L.) Accessions Grown in Mississippi. *J. Agri. Food Chem.*, 2007; 56: 241-245.
12. Nyak V and Uma DP. Protection of mouse bone marrow against radiation-induced chromosome damage and stem cell death by *Ocimum* flavonoids Orientin and vicenin. *Radi. Res.*, 2005; 163: 165-171.
13. Opalachenova G and Obreshkova D. Comparative studies on the activity of basil-an essential oil from *Ocimum basilicum* L.-against multidrug resistant clinical isolates of the genera *Staphylococcus*, *Enterococcus* and *Pseudomonas*. *J. Micro. Method.*, 2003; 54: 105-10.
14. Sahoo Y, Pattnaik SK and Chand PK. *In vitro* clonal propagation of a medicinal plant *Ocimum basilicum* L. (Sweet basil) by axillary shoot proliferation. *In Vitro Cell. Dev. Biol. Plant*, 1997; 33: 293-296.
15. Sappakul P, Miltz J, Sonneveld K and Bigger SW. Antimicrobial properties of basil and its possible application in food packaging. *J. Agri. Food Chem.*, 2003; 51: 3197-3207.
16. Chopra RN, Nayar SL and Chopra IC. Glossary of Indian Medicinal Plants (Including the Supplement). Council of Scientific and Industrial Research, New Delhi. 1986; pp 256.
17. Martin KW and Ernst E. Herbal medicines for treatment of fungal infections: a systematic review of controlled clinical trials. *Mycology*, 2004; 47: 87-92.
18. Adiguzell A, Glluce M, Sengul M, Ogutcu H, Sahin F and karaman I. Antimicrobial Effects of *Ocimum basilicum* (Labiatae) Extract. *Turk. J. Biol.*, 2005; 29: 155-160.
19. Kaya I, Yigit N and Benli M. Antimicrobial activity of various extracts of *Ocimum basilicum* L. and observation of the inhibition effect on bacterial cells by use of Scanning Electron Microscopy. *African J. Trad. Compl. Altern. Med.*, 2008; 5: 363-369.
20. Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physio. Plant*, 1962; 15: 473-497.
21. Azoro C. Antibacterial activity of crude extract of *Azadirachita indica* on *Salmonella typhi*. *World J. Biotech.*, 2000; 3: 347-351.
22. Collins CH. Microbial. Method. London, Butterworths. 1967; pp 335.
23. Dode LB, Bobrowski VL, Braga BJE, Seixas FK and Schuch MW. *In vitro* propagation of *O.basilicum* L. (Lamiaceae). *Maringa*, 2003; 25: 435-437.
24. Omoregbe RE, Ikube OM and Ihimire IG. Antimicrobial activity of some medical plants extract on *Escherichia coli*, *Salmonella paratyphi* and *Shigella dysenteriae*. *African J. Med. Sci.*, 1996; 25: 373- 375.
25. Goleniowski M and Trippi VS. Effect of growth medium composition on psilostachyinolides and altamicine production. *Plant Cell Tis. Org. Cult.*, 1999; 56: 215-218.
26. Takahashi D and Fjita Y. In: 'Plant cell culture in Japan'. (Eds. Komamine A, Misawa M, DiCosmo F). *Cosm. Mater.*, 1991: 72-78.