Optimization of antibacterial activity of ethanolic extracts of *Eucalyptus tereticornis* and *Nigella sativa:* Response surface methodology

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Abstract: The screening of plants for medicinal purposes represents an effort to discover newer, safer, and possibly more effective drugs. Design of the present study was made aiming to the optimization of the antibacterial activity of ethanolic extracts of *Eucalyptus tereticornis* (leaves) and *Nigella sativa* (seeds) against bacteria belongings to both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) spectrum by using response surface methodology. 20 g powder of each *E. tereticornis* (leaf) and *N. sativa* (seeds) were mixed with 200ml of ethanol at room temperature, and then it was centrifuged at 4000 rpm for 10 min to separate the supernatants, and allowed to dry in order to obtain ethanol free extracts. A fresh bacterial culture of 100µl of test microorganism was inoculated onto media and spread homogeneously. The antimicrobial activity of ethanolic extracts showed that all the concentrations tested were effective against the test microorganisms. The diameters of zones of inhibition exhibited by *S. aureus* PCSIR-83 were in the range of 0-28mm, *E. coli* PCSIR-102 (0-28mm) and *B. subtilis* PCSIR-05 (15-26mm). The combination of *N. sativa* (15mg/µl) and *E. tereticornis* (20mg/µl) were found most effective at pH 9.0 and temperature 35°C. Our results clearly indicate that Gram positive bacteria showed more sensitivity than Gram-negative bacteria.

Keywords: *Eucalyptus tereticornis, Nigella sativa,* antimicrobial agents, response surface methodology.

INTRODUCTION

In spite of the availability of a number of antibiotics, globally infectious diseases are still responsible for causing approximately 50,000 deaths every day. In past few decades, several types of antibacterial agents have been synthesized and employed for the treatment of infectious and communicable diseases most often caused due to microorganisms (Ahmad and Beg 2001). However, use of antibacterial drugs have raised major concern worldwide as misuse and prolong use of synthetic antibiotics often leads to the appearance and transmission of microorganisms resistant to multi drug. These limitations emphasize the need for focusing towards the identification and development of new antimicrobial drugs from various sources more importantly from natural product such as medicinal plants (Cordell 2000). There are many reports in the literature describing studies of different type of plants such as herbs, shrubs and trees with the aim of knowing their phytoconstituents and to make good use of them to treat various diseases to replace many existing drug compounds to which many microorganisms have already developed resistance. The screening of plants for medicinal purposes represents a serious effort to discover newer, safer, and possibly more effective drugs having potential to fight pathogenic bacteria and fungi (Alviano and Alviano 2009). These antibacterial substances isolated from plants appear to be one of the important alternative approaches to combat antibiotic resistance and in the management of diseases. The green medicines are widely believed as safe and dependable in contrast with expensive synthetic drugs that have undesirable or side effects along with beneficial effects (Karou *et al*., 2005).

The plants have been in use in traditional medicine since long time but are still understudied, particularly in clinical microbiology (Kupeli *et al*. 2007). Scientists have always shown a great deal of interest in the biologically active compounds of the medicinal plants. In past few decades, the curiosity in plants possessing antibacterial, antifungal, anti-inflammatory activity for curing various diseases has grown many folds (Arora and Kaur 1999). Several studies have established that in many plants contains substances like peptides, tannins, alkaloids, essential oils, phenols, and flavonoids among others, which have potential to be

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served as sources for antimicrobial production. These substances or compounds are of great significance in therapeutic application against different human pathogens including bacteria and fungi. New inventions in the arsenal of agents available to treat infections can no longer match the accelerating number of antibiotic resistant bacteria. It can be suggested from the literature reports and ethnobotanical records that the plants are the sleeping giants of pharmaceutical industry (Okigbo and Omodamiro 2007; Takahashi *et al*. 2004). *Eucalyptus tereticornis* belongs to the family of *myrtacea* and it is known as "forest red gum". The medicinal usefulness of the red gum tree has been the subject of numerous studies. Some of the reported phytoconstituents of the tree included essential oils, sterols, alkaloids, glycosides, flavonoids, tannins, and phenols. The tree has been always widely used in traditional medicine throughout the world for treating diseases covering a wide range including colds, coughs, asthma, diarrhea, dysentery, hemorrhage, laryngalgia, laryngitis, sore throat, spasm, trachagia and vermifuge and a decoction of this plant has been used to treat enteric infections including diarrhea and dysentery, constipations and other stomach problems (Sadlon and Lamson 2010; Maurya and Srivastava 2012). The main component found in *Eucalyptus* essential oil of most species is 1,8-cineole, representing about 70% of the total oil by gas chromatography (Ali and Blunden 2003).

After extracting the essential oil, the leaves of *E. tereticornis* can be further processed for large scale isolation of anticancer agent, ursolic acid and ursolic acid lactone (Javed *et al*. 2012). *Nigella sativa* is a member of the family ranunculaceae and is known by the common names kalonji (Urdu), black seed or black cumin (English) and habat-baraka (Arabic). It is cultivated in various parts of the world as a spicy plant (Yakoot and Salem 2011). *Nigella sativa* is the black seed that the Prophet Mohammed (PBHU) has referred to have healing powers that he told "there exists, in the black grains, health care of all the diseases, except death". The Holy Bible also described it as the curative black cumin and it is also named as the Melanthion of Hippocrates and Discroides and as the Gith of Pliny (Landa *et al*. 2009; Ezoubeiri *et al*., 2005). The chemical constituents presence in the seeds of *N. sativa* are more than one hundred. The constituent of *N. sativa* seeds which is regarded, as most antibacterial is thymoquinone. Other ingredients include dithymoquinone, nigellone, thymohydroquinone, ascorbic acid (vitamin C), tocopherol (vitamin E), linoeic acid, oleic acid, carvacrol, lipase, tanethole and 4-terpineol (Yakoot and Salem 2011). The organic extracts of several species of *Nigella* have been recognized to have inhibitory power activity against broad spectrum of bacteria, both Gram-positive and Gram-negative (Smania Jr *et al*. 1999).

Objectives

This study aims to analyze and optimize the antibacterial activity of ethanol extracts of *N. sativa* seeds and *E. tereticornis* leaves using surface response methodology.

MATERIALS AND METHODS

Sample collection and extraction

Fresh leaves of *E. tereticornis and N. sativa* were collected from the vicinity of University area. The plants were identified by expert taxonomist from the Botany Department, University of Lahore. Leaves were washed gently under tap water and left to dry at room temperature for 2 days, the leaves were then crushed separately to make powder. 20 gram of the powder of *E. tereticornis and N. sativa* were mixed separately with 200 ml ethanol in conical flasks. The flasks containing extracts were heated on water bath for 1h and placed at room temperature for 5 days. The flasks were manually shaken daily to obtain maximum extraction. After 5 days, each extract added to falcon tubes and centrifuged at 4000 rpm for 10 min to separate the supernatant. The supernatant containing extracts of *E. tereticornis and N. sativa* were transferred into pre-weighed beakers and were left to dry completely on water bath at 60°C to obtain an ethanol free extract residue of *E. tereticornis and N. sativa*.

Preparation of stock solutions

Two stock solutions were prepared by dissolving appropriate amount of the powdered extract of *E. tereticornis* in dimethyl sulfoxide (DMSO) to get final concentration of 10mg/µl and 20mg/µl. Oily extract of *N. sativa* was maintained at 7.5mg/µl and 15mg/µl in DMSO.

Test microorganisms

In vitro antimicrobial activity was examined for the ethanol extracts of *N. sativa* and *E. tereticornis* against three bacteria. Pakistan Council of Scientific and Industrial Research (PCSIR) laboratories, Lahore provided the microorganisms. The strains were *Staphylococcus aureus* (PCSIR–83), *Bacillus subtilis* (PCSIR-05), and *Escherichia coli* (PCSIR–102). Microorganisms were maintained on nutrient agar slants at 4°C.

Inoculum preparation

Inoculum of a loop full of test organism in Muller Hinton broth (Oxoid, UK) and incubation for 24h at 37±1ºC was performed. Comparison between the bacterial cultures from fresh media and 0.5 McFarland turbidity standard, which is equivalent to approximately 1×10^5 CFU/ml was done. To obtain the inoculum, the suspension was diluted 100 times with Muller Hinton broth to produce 1×10^6 colony forming units (CFU)/ml were used in all the experimentation (Uniyal *et al*. 2006).

Table 1: Antimicrobial activity of extracts of *N. sativa* and *E. tereticornis* against different microorganisms

Run	Variable				Antimicrobial activity in terms of diameter of zone of inhibition (mm)		
	pH	Temp.	*N. sativa* (mg/µl)	*E. tereticornis* (mg/µl)	*S. aureus* PCSIR-83	*B. subtilis* PCSIR-05	*E. coli* PCSIR-102
1	9.0	35.0	0	20	18	16	17
2	6.0	40.0	15	0	–	25	16
3	9.0	40.0	0	0	0	0	0
4	6.0	37.5	7.5	10	–	16	20
5	9.0	37.5	7.5	10	22	19	18
6	6.0	35.0	0	0	0	0	0
7	6.0	40.0	0	20	–	15	20
8	6.0	35.0	15	20	–	26	27
9	7.5	37.5	0	10	18	15	16
10	6.0	35.0	0	20	–	17	20
11	7.5	37.5	7.5	10	20	18	17
12	9.0	40.0	0	20	17	16	17
13	7.5	37.5	7.5	20	23	22	20
14	6.0	35.0	15	0	0	24	0
15	7.5	37.5	15	10	25	23	21
16	7.5	37.5	7.5	0	18	15	13
17	9.0	40.0	15	20	23	21	18
18	9.0	35.0	0	0	0	0	0
19	6.0	40.0	0	0	0	0	0
20	6.0	40.0	15	20	–	23	12
21	7.5	40.0	7.5	10	21	19	17
22	9.0	35.0	15	0	27	16	12
23	7.5	35.0	7.5	10	21	19	17
24	9.0	40.0	15	0	28	15	13
25	9.0	35.0	15	20	26	22	28

= No zone of inhibition (Resistant)

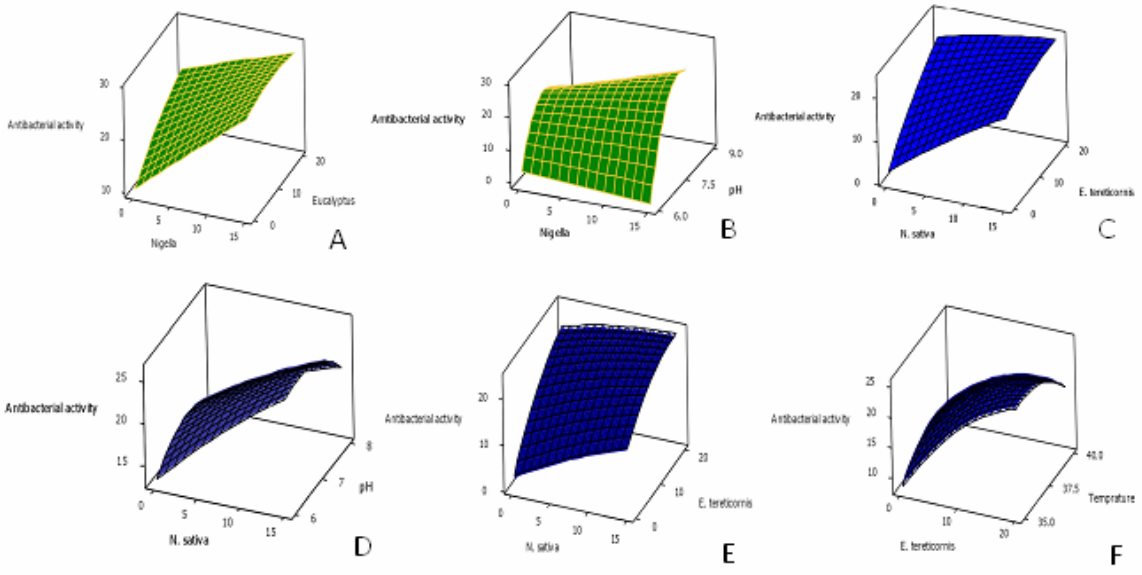

Fig. 1: Response surface plots showing the antibacterial activity of: (A) *E. tereticornis* and *N. sativa* against *S. aureus*, pH 7.5, Temp. 37.5°C; (B) *N. sativa* at different pH against *S. aureus*, Temp. 37.5°C; (C) *E. tereticornis* and *N. sativa* against *B. subtilis*, pH 7.5, Temp. 37.5°C; (D) *N. sativa* at different pH against *B. subtilis*, Temp. 37.5°C; (E) *E. tereticornis* and *N. sativa* against *E. coli*, pH 7.5, Temp. 37.5°C; (F) *E. tereticornis* at different temperatures against *E. coli*, pH 7.5.

Table 2: Regression coefficients for the optimization of antibacterial activity of *N. sativaand E. tereticornis* against selected bacteria

Term	Coefficient	P-value
1: *S. aureus* PCSIR–83		
Constant (Intercept)	26.7929	0.000
PH*PH	−24.0318	0.001
*N. sativa***E. tereticornis*	−0.6406	0.015
*Nigella**PH	3.2344	0.001
r^2 = 96.11% r^2 (pred.) = 68.30% r^2 (adj.) = 90.67%		
2: *B. subtilis*–05		
Constant (Intercept)	22.3724	0.000
N. sativa	1.7042	0.046
PH	−5.5540	0.033
*N. sativa***E. tereticornis*	−0.8125	0.000
*N. sativa**PH	−1.1250	0.020
r^2 = 96.87%, r^2 (pred.) = 81.86%, r^2 (adj.) = 92.48%		
3: *E. coli*–102		
Constant (Intercept)	23.0320	0.000
*E. tereticornis**Temp	−1.3125	0.043
r^2 = 87.40%, r^2 (pred.) = 0.63%, r^2 (adj.) = 69.75%		

Media preparation

Mueller Hinton Agar (MHA) media (Oxoid, UK) was prepared by suspending 38 g in 1000 ml of distilled water. To sterilize the media autoclaving at 121°C for 15 min was done. The media was transferred into sterile Petri plates at around 50°C. To observe the effect of pH on the growth of tested bacteria, pH was adjusted by adding 0.1M HCL or 0.1M NaOH into the media. The pH was adjusted at 6, 7.5, and 9 with the help of digital pH meter.

Agar well diffusion assay

The antibacterial activity of ethanol extracts of *E. tereticornis and N. sativa* was examined using the agar well diffusion assay as described earlier (Rakotonirainy and Lavédrine 2005). MHA media was poured into petri plates of 90 mm diameter to solidify to make a base layer. The MHA plates were marked to divide into required parts and labeled for specific organism, extract name, and pH. A fresh bacterial culture of 100 µl of each test microorganism was inoculated onto MHA media and spread homogeneously using a glass spreader, incubated for 15 min at 37°C to complete dryness of media surface. Wells of 6 mm in diameter were punched off with the help of sterile borer in MHA. Then wells were filled with different quantities of ethanol extracts singly or in combination. Petri Plates were placed in refrigerator for 30 min for diffusion of extracts and then incubated at different temperatures ranging from 35°C-40°C±1°C for 24 hours. At the end of the incubation period, the zone of inhibition (including well diameter) was measured. Ciprofloxacin 5 µg/disc was employed as positive control and DMSO (solvent) employed as negative control. Ciprofloxacin was employed as standard due to its antibacterial activity for the said strains.

Experimental design and statistical analysis

The extract of *N. Sativa* and *E. Tereticornis*, pH and temperature have been predictive to play an important role to affect the growth of bacteria were selected as main variable to optimize the process. Experimental range and level of independent variables such as *N. sativa* extract (X_1), *E. tereticornis* extract (X_2), pH (X_3) and temperature (X_4) was studied at different levels (7.5mg/µl-15mg/µl) for *N. sativa* extract, (10mg/µl-20mg/µl) for *E. tereticornis* extract, pH 6.0, 7.5 and 9.0 and temperature 35, 37.5 and 40°C. Total 25 runs (a series of tests) in three blocks were done to optimize the antibacterial activity of the extracts. Block variables were run in three different incubators for three different runs at the same time. Upon completion of experiments, antibacterial activity was taken as a dependent variable or response. Minitab statistical software release 15 was used for analyzing ting of data and generating of response surface graphs. The graphical representation of the model equation leads to the response surface plots which represent the individual and interactive effects of test variables on the response. A second order full model including interactions was fitted to the antimicrobial activity data after running the experiments and measurement of the activity levels.

RESULTS

Antibacterial activity of the ethanolic extracts

The ethanolic extracts of *E. tereticornis* and *N. sativa* were tested for their antibacterial activity singly or in combination with different concentrations ranging from 7.5 mg/µl–35 mg/µl and at varying pH and temperature. The tested plants have been in use as folk medicine and were familiar to the local people of Punjab, Pakistan. The outcomes of the antimicrobial activity of medicinal ethanolic extracts showed that all the concentrations were effective against tested microorganisms with varying zones of inhibition. The diameter of zones of inhibition exhibited by *S. aureus* PCSIR-83 were in the range of 0-28 mm, *E. coli* PCSIR–102 (0-28mm) and *B. subtilis* PCSIR-05 (15-26mm). At pH 6.0 and temperature 35°C, the ethanolic extract of *E. tereticornis* when tested alone at concentration 20mg/µl was found ineffective to *S. aureus* PCSIR–83, however, it was effective in inhibiting the growth of *B. subtilis* PCSIR–05 (17mm zone diameter) and *E. coli* PCSIR-102 (20mm). *N. sativa* extract when tested alone at 15mg/µl concentration showed no inhibitory effect on *S. aureus* PCSIR–83 and *E. coli* PCSIR–102 at pH 6.0 and temperature 35°C, but it was found antibacterial against *B. subtilis* PCSIR–05 (24 mm). *S. aureus* PCSIR–83 was again found resistant when the extract of *N. sativa* (15mg/µl) and *E. tereticornis* (20mg/µl) were tested in combination. However, this combination was strongly antibacterial against *B. subtilis* PCSIR–05 (26mm) and *E. coli* PCSIR–102 with zone diameter of 27 mm (table 1). At pH 7.5 and temperature 37.5°C, the combination of *N. sativa* (7.5 mg/µl) and *E. tereticornis* (20mg/µl) showed effective antibacterial activity against all three bacteria tested. When *N. sativa* extract (7.5mg/µl) and *E. tereticornis* (10 mg/µl) were tested in combination at pH 7.5, the temperature of 35°C and 40°C had no effect on the antibacterial activity. The combination of *N. sativa* (15 mg/µl) and *E. tereticornis* (20mg/µl) was found most effective at pH 9.0 and temperature 35°C (table 1).

Optimization of ethanolic extracts for antibacterial activity using response surface methodology

The Response Surface Methodology (RSM) is the mathematical and statistical techniques to model and analyze of problems where a response of interest is influenced by several variables and the aim is to get optimal response. The response surface methodology has been widely and successfully applied in various fields. The current study involves the optimization of the ethanolic extracts for maximum antibacterial activity against selected bacterial strains having with interaction between four variables. After the analysis of response data and treatment combination was done, the least square estimate of parameters (β) of the final model were determined and it was concluded that among four interaction terms the pH and extracts of *N. sativa* and *E. tereticornis* were significant. The significant term can be seen in p-values, which are less than 0.05 (table 2). The calculation of the regression equation coefficients was done and the data was fitted to a second-order regression equation. The response of antibiotic activity (Y) can be expressed in terms of the following regression equation:

$$Y=\alpha + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4$$

To study the interaction among various factors used and to find out determine optimum level of each factor for maximum zones of inhibition from antimicrobial activity required the plotting of the three dimensional response surfaces (fig. 1 A-F). However, to understand the interaction behavior of parameters, the response surfaces were investigated considering each couple of variables while the other factors remained constant. The *P*-values acted as a tool for checking the significance of the interaction effects. The quality of fit of regression model was expressed via the coefficient of determination (r^2) and the adjusted r^2. The linear model was used to fit the experimental results and the following expression was obtained by putting the value of coefficient for *S. aureus*–83 final model:

$$Y=\alpha + \beta_3X_3^2 + \beta X_1X_2 + \beta X_3X_1$$

$$Y= 26.7929 - 24.0318(X_3^2) - 0.6406(X_1X_2) + 3.2344(X_3X_1)$$

Experimental results for *B. subtilis*–05 when fitted to a linear model, the following equation was obtained:

$$Y=\alpha+ \beta_1X_1 + \beta_3X_3 + \beta X_1X_2 + \beta X_1X_3$$

$$Y=22.3724 + 1.7042(X_1) - 5.5540(X_3) - 0.8125(X_1X_2) - 1.1250(X_1X_3)$$

Similarly, for *E. coli*–105, the following expression was obtained by putting the experimental values in the regression equation:

$$Y=\alpha + \beta X_2X_3$$

$$Y=23.0320 - 1.3125(X_2X_3)$$

DISCUSSION

In present study, the ethanolic extracts of *N. sativa* and *E. tereticornis* have been found possessing antibacterial activity against test microorganisms. The tested plants have been in use as folk medicine and are familiar to the local people of Punjab, Pakistan. Many plants contain potentially useful substances, which can be used as alternative chemotherapeutic agents. The *in vitro* antibacterial activity assay is the very first step to achieve this goal. Large varieties of medicinal plants have been screened and many of them have been proven to possess antibacterial or antifungal activity (Alma *et al*. 2004). Therefore, it is a challenge to seek the *in vitro* antimicrobial activity of natural compounds from these ethno medicinal plants on pathogenic bacteria. The ability of the extracts of *Eucalyptus* species inhibit the growth of both Gram-positive and Gram-negative bacteria has been widely reported (Sartorelli *et al*. 2007). Flavonoids and alkaloids from different species of *Eucalyptus* plants are known to have antimicrobial activity (Mulyaningsih *et al*. 2010; Elaissi *et al*. 2011). Medicinal properties of

Eucalyptus species have been screened and investigated and it has been in use throughout worldwide folk medicine. The *Eucalyptus* plants have been reported as an important source of essential oils possessing biological activities of wide range such as antibacterial, antifungal, analgesic and anti-inflammatory properties (Gilles *et al*. 2010) and a long history of application in treatment of colds, influenza, other respiratory infections, rhinitis, and sinusitis (Maurya and Srivastava 2012). These oils can be found in the leaves of more than 300 species of *Eucalyptus* (Bachir and Benali 2012). The antimicrobial properties of the *Eucalyptus* essential oils are demonstrated a number of studies and other extracts against a wide range of microorganisms (Bachir and Benali 2012; Salman *et al*. 2008). The major constituents of Eucalyptus essential oil and extracts have been reported to be cineole, limonene, cymene, terpinene, pinene and terpineol and several other components were found in small amounts (Gilles *et al*. 2010; Bachir and Benali 2012). Bachir and Benali conducted research on the effect of essential oil from *Eucalyptus globulus* on *E. coli* and *S. aureus* and observed that the essential oil shows antibacterial activity for the tested bacteria (Hannan *et al*. 2008). Elaissi and coworkers found antibacterial activity of the essential oil from different species of *Eucalyptus* plant against a variety of Gram-positive and Gram-negative bacteria such as *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 227853, *Enterococcus faecalis* ATCC 292112, *Staphylococcus aureus* ATCC 25932 (Bachir and Benali 2012). In another study, *Pseudomonas aeruginosa* MJH 4 and MJH 207 have been found inhibited by essential oils and extracts from *Eucalyptus globules* (Ali and Blunden 2003).

The antibacterial and antifungal activity of black seeds (*Nigella sativa*) and its extracts have been demonstrated by several research groups (Salem *et al*. 2010). In a study, investigation of antibacterial activity of *N. sativa* extracts against clinical isolates of methicillin resistant *Staphylococcus aureus* was performed. All tested methicillin resistant strains of *S aureus* showed sensitivity to ethanolic extract of *N. sativa* at the concentration of 4 mg/disc with MIC range of 0.2-0.5mg/ml (Chaieb *et al*. 2011). Salem and colleagues found that the antibacterial activity of *N. sativa* seeds was similar to the triple therapy against *Helicobacter pylori* (Wajs *et al*. 2008). The biofilm inhibition and antibacterial activity of thymoquinone, which is a major constituent of *N. sativa* seeds, were tested on 11 human pathogenic bacteria. Thymoquinone demonstrated a significant bactericidal activity against wide range of human pathogenic bacteria particularly *S. aureus* ATCC 25923 and *S. epidermidis* CIP 106510 (Gali-Muhtasib *et al*., 2006).

The black seeds are found to be rich in diverse chemical composition. The nigellin, metarbin, melanthin, anthraquinones, glycosides, saponines, volatile oils, fixed oils, tannins, p-cymene, limonene, γ-terpinene, α-thujene, carvacrol, α-pinene, β-pinene, thymoquinone, α-thujene, longifolene are some of the major constituents of *N. sativa* essential oil (Abdulelah and Zainal-Abidin 2007). Apart from these ingredients, *N. sativa* seeds also contain 15 amino acids, proteins, carbohydrates, alkaloids, crude fibre, and minerals, like calcium, iron, sodium and potassium (Cojocaru and Zakrzewska-Trznadel 2007). The results obtained from study may justify and support the application of extracts of these plants in traditional medicine for the treatment of certain infections. In the past two decades, there was dramatic increase of the studies on effect of *N. sativa* seed extract or its active compound (thymoquinone) on different body systems *in vivo* and *in vitro* (Myers *et al*. 2004; Yakoot and Salem 2011). The results from the experiment clearly showed an important antibacterial activity of the *N. sativa* and *E. tereticornis* extracts with clear zones of inhibition against the bacteria tested. This experiment has further suggested that Gram-positive bacteria are generally more sensitive to the extracts than Gram-negative bacteria. The difference in sensitivity shown by Gram-positive and Gram-negative bacteria to plant extracts, might be due to the morphological differences. An outer phospholipidic membrane carrying structural lipopolysaccharide is present in Gram-negative bacteria making the cell wall impermeable to organic solutes, while porins make a barrier to the aqueous solutes. The Gram-positive bacteria in this regard should be more sensitive since they lack this outer layer (Kaushik *et al*. 2006).

We found in the present study that combination of the extracts of *E. tereticornis* and *N. sativa* were more active in inhibiting the growth of test bacteria than the extracts when tested alone.Clinically it is expected to achieve success if the amalgamations of antimicrobials exhibit *in vitro* synergism against infecting microbial strains. Thus, results from *in vitro* synergism can be of great value selecting of most favorable antimicrobials combinations for the practical issues of serious bacterial infections (Kaushik *et al*. 2006). Antimicrobial agents used in different combinations might enhance the effectiveness of each agent while optimal efficacy being achieved using a lower dose of each drug. Pharmacological benefits could be increased as different drugs would clear infection from different body systems. In third world countries like Pakistan where contagious diseases are common, it is important to search out and promote plant-derived medicines.

The Response Surface Methodology (RSM) is important for the statistical design of the experiment can be defined as a collection of mathematical and statistical techniques useful for the modeling and analysis of problems where a response of interest is modified by several variables and the objective is to get optimum response. RSM is most extensively applied in the situations where several input

variables potentially influence some performance measure or quality characteristic of the process (Chen *et al*. 2009). In recent years, fields such as biology, medicine, and economy (El-Naggar and Abdelwahed 2014) have been showing successful applications of RSM. The present study involves the optimization of the ethanolic extracts for maximum antibacterial activity against selected bacterial strains having with interaction between four variables. At the completion of the analysis of response data and treatment combination, the least square estimate of parameters (β) of the final model were determined and it was concluded that among four interaction terms the pH and extracts of *N. sativa* and *E. tereticornis* were significant. The *P*-values were used as a tool to verify the significance of the interaction effects. The quality of regression model fitness was expressed via the coefficient of determination (r^2) and the adjusted r^2. In our results, the r^2 values were always between 0 and 1. As the value ofr^2get closer to 1, the model becomes stronger, and the prediction of the response gets better. The antibacterial activity of the extracts against *E. coli*–102 was not well explained by the RSM as the r^2 value for *E. coli*–102 was found low (0.8740). Ther^2 value of a regression model is higher than 0.9 indicated high correlations. Therefore, ther^2 value presented by this study fit well between the observed and predicted responses, and implies that the model is reliable for *S. aureus* and *B. subtilis* in the present study. In addition, the high value of the adjusted determination coefficient (Adj. r^2=0.9067 for *S. aureus* and 92.48 for *B. subtilis*) indicates a high significance of the model. A good correlation between the experimental and predicted values is indicated by the higher value of the r^2. Thus, the analysis of the response trend using the model was considered to be reasonable.

CONCLUSION

It may be concluded from this study that *in vitro* analysis of the extracts from fallen leaves of *Eucalyptus tereticornis* and *Nigella sativa* seeds exhibited significant zones of inhibition against *S. aureus, B. sutilis and E. coli*. These encouraging results suggest that these ethnomedicines have great antimicrobial potential, and may be exploited as natural antibiotics for treating several infectious diseases. The synergistic effect provided by the association of different plant extracts against pathogenic bacteria causes new choices for the treatment of infectious diseases to emerge. To standardize methods of extraction and *in vitro* testing would be advantageous to make the search more systematic and interpretation of results would be facilitated.

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