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Effect of Ethanolic Extracts of Spices Named *Nigella sativa, Allium sativum and Syzygium aromaticum* Against *Pseudomonas aeruginosa in Vitro*

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Abstract: Background a wide range of plants and spices having the medicinal properties have been explored and used in so many researches worldwide for their antimicrobial activity against pathogenic bacteria, fungi and viral infections. Pathogenic bacterial infections specially the Multi drug resistant *Pseudomonas aeruginosa* have become a major problem worldwide so new antimicrobial agents are essentially needed to overcome this problem. Extracts of black seeds *Nigella sativa, Allium sativa and Syzygium aromaticum* have been used in traditional medicines for long. The aim of this study was to focus on effect of antibacterial activity of these three medicinal spices extracts on inhibitory activity against the *Pseudomonas aeruginosa*. *Material and Methods*: ethanolic extracts of these three spices were screened and evaluated against P. aeruginosa. The antibacterial inhibitory activity was determined by Kirby Bauer Agar Well diffusion method and agar disc diffusion method for dilutions 10^{-1}, 10^{-2}, 10^{-3} (Conc. 100mg/mL, 10mg/mL, 1mg/mL respectively) using Mueller Hinton agar. *Results:* obtained by *P. aeruginosa* with these extracts were particularly interesting, it showed inhibition zone of >15mm for *Nigella sativa* and *Allium sativa*, ≤15 mm for *Syzygium aromaticum* at dilution 10^{-1} (Conc. 100mg/mL)., ≤15 mm for *Nigella sativa, Allium sativa*, ≤07mm *Syzygium aromaticum* respectively at dilution 10^{-2} (conc. 10mg/mL) and for *Nigella sativa, Allium sativa* at dilution 10^{-3} (1mg/mL), but, Nil for *Syzygium aromaticum* at dilution 10^{-3} *Conclusion*: ethanolic extracts of *Nigella sativa* is more effective antibacterial agent against *P.seruginosa* in comparison to *Allium sativa* and *Syzygium aromaticum* at higher conc of 100mg/mL, showing significant correlation b/w conc of extract and inhibition zone. The highest remarkable inhibition was observed with ethaonolic fraction of Nigella sativa 10^{-1} (Conc. 100mg/mL). The growth of *P.aeruginosa* visibly inhibited to *Allium sativa* extract and to *Syzygium aromaticum* 10^{-2} while not *S.aromaticum* at dilution 10^{-3}; showed non inhibitory effect against *P.aeruginosa*.

Key words: Spices (*Nigella sativa* · *Allium sativa* · *Syzygium sativa*) Extracts · Antibacterial Activity · Multi Drug Resistant *Pseudomonas aeruginosa* · Kirby Bauer Agar Well Diffusion · Agar Disc Diffusion · Zone Of Inhibition

INTRODUCTION

Pseudomonas aeruginosa, among the other members of bacterial genus Pseudo, is the major nosocomial pathogen due to its ubiquitous nature and ability to survive and colonize in hospital reservoirs. It's a major cause of infections in clinically ill, immunocompromised and in hospital staying patients. Aloush *et al*. [1] *P.aeruginosa* is a pathogen that has a high level of intrinsic resistance to synthetic antimicrobial drugs and has ability to become even more drug resistant [2]. The versatility and ability of *P. aeruginosa* to combine different resistance mechanisms has led to emergence of strains that are resistant to multiple antibiotic drugs which severely limits therapeutic options for treating infections [3, 4]. Interim definitions of *P. aeruginosa* have been recently reported [5]. The outbreak in hematology department of a hospital in Spain caused by *P. aeruginosa* that produced extended spectrum resistant to beta lactamases [6]. The use of synthetic drugs that are

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biochemically and genetically modified so not reliable for many controversial issues as expensive, inadequate and often have issues with adulterations and side effects [7]. So other antimicrobial agents are urgently needed to cope this problem of increasing number of pathogens [8]. Herbal extracts of spices and herbs have been used for long due to their antimicrobial activities against pathogens [9]. Thus scientists are challenged to new ideas of alternative herbal ethnomedicine and drugs to beat the resistant pathogens. Studies have reported the active phenolic compounds in spices and herbs significantly contributed to their pharmaceutical and bactericidal properties [10-12]. The use of herbal extract products as medicines can be traced as far back as beginning of human civilization. Nature has blessed us a very rich botanical wealth including so many medicinal plants. Herbal medicine is mainstay of 75-80% of whole population as a major traditional therapy by the use of plant extract and active constituents [13, 14]. Medicinal plants have ability to inhibit growth of wide range of pathogenic microbes due to essential oils in their extracts [15, 16]. *Nigella sativa*, an herbaceous plant, is commonly known as black seed which belongs to botanical family of Rannuculaceae. *N.sativa* seeds have been used for nutritional and medicinal purposes in many countries [17, 18]. *N. sativa* black cumin is the black seed medicine for every disease except death, its oil in combination with drug is used to treat helminthic parasites in ruminants [19, 51]. The black seeds contain 36-38% fixed oils with proteins, alkaloids, saponins and essential oils which have been reported to possess antimicrobial activity and stimulatory effect on immune system [20-22]. Black cumin seeds, active compound is nigellone, thymollone have been reported to exhibit many pharmacological effects that includes antiparasitic, antibacterial, antifungal, antiviral and anti-inflammatory activities [23]. Clove (*Syzygium aromaticum*, syn. *Eugenia aromaticum, Caryophyllus aromaticus, Eugenia caryophyllata*) are the aromatic dried flower buds of a tree in a family Myrtaceae [24]. Cloves are commonly used in Chinese medicine named Ayurveda and in western herbalism. Cloves are being extensively used as Carminative to increase hydrochloric acid in stomach and to improve peristalsis [25]. Also have use in dentistry where essential oil Eugenol as Anadyne for dental emergencies [26]. Antimutagenic and antibacterial [17, 28]. The more dose of clove oil is toxic to human cells showing life threatening consequences as hepatic failure, CNS disorderand lethal oral dose is 3.752g/Kg body weight [29]. *Allium sativa* (Garlic) has been used worldwide as a traditional medicine for >4000 years to treat several disorders such as arthritis, diabetes and infectious diseases e.g. malaria, common cold, tuberculosis [30]. Further Microbiologist Louis Pasteur demonstrated the bactericidal properties of garlic; active compound is allicin, later it was called "Russian Penicillin" in 2nd world war. In Africa particularly in Nigeria used to treat abdominal discomfort, diarrhea, otitis media and resp. tract infections [31]. The project under study aimed to screen and evaluate the antibacterial activity of all three spices extracts against *P. aeruginosa* and to compare which one can be used effectively as ethnomedicine against this bacterium and ultimately results in correlation b/w concentration of spices and zone of inhibition.

MATERIALS AND METHODS

Preparation of Bacterial Sample: The bacterium *Pseudomonas aeruginosa* pure culture was obtained from Microbiology department, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences Lahore. The bacterium was confirmed on colony structure, colony morphology, Gram staining, pigment production, special odor and oxidase test.

Maintenance of Bacterium Culture: The bacterium was cultured on Nutrient agar (Merck) at 37°C for 24 hours in laboratory aerobic incubator. The culture stored at 4°C.

Preparation and Ethanolic Extraction of Spices Samples: *Nigella sativa* black cumin seeds, five *Allium sativum* bulbs and an amount of *Syzygium aromaticum* were purchased from a local herbal market. 150g of each spice was washed 1st with tap water then thoroughly wash with distilled water thrice followed by washing with 70% ethanol thrice. All were dried in laboratory egg incubator at 45°C overnight. Grind each spices individually in sterile pestle and mortar clearly crushed to form powder. Sieve through gauze layers to attain fine powder then it was soaked in 200mL of 95% ethanol. Wrap each of the three flasks with Aluminium foil and place in lab incubator at 37°C for 5 days to make 100% concentrated extract of powdered spices. Shake once in each of the five days. On post incubation, filter through Whatmann filter paper no.1. The filtrate is considered as 100% conc. Evaporation of filtrate through rotary evaporator, finally extract comes in powder form [32, 33].

Antibacterial Sensitivity Testing

Kirby Bauer Agar Well Diffusion Method: Mixed 1g of each of three spices extract powdered form in 1mL of 95% Ethanol. Then, three serial dilutions 10^{-1} (100mg/mL), 10^{-2} (10mg/mL), 10^{-3} (1mg/mL) were made in distilled water. Overnight confluent lawn of *P. aerogenosa* was formed on Muller Hinton Agar (15-20mL/plate) and serial dilutions all the three spices extract was tested against the confluent culture by disc diffusion and well diffusion assays. Properly labeled the plates after inserting the ethanol extract in three wells and sterile PBS in 4th one as a negative control and incubated at 37°C for 24 hours [34, 35].

Agar Disc Diffusion Method: Discs made of sterile filter paper Whatmann #01 soaked in each of three dilutions of an extract applied on each of three plates of MH agar having confluent lawn of 24 hours old culture of *P.aeruginosa*, after drying the discs with 4th one PBS soaked dried disc as a negative control. Incubate at 35-37°C for 24 hours. On post incubation observe the plates for inhibition patterns of extracts [36, 50].

Statistical Analysis: Bivariate correlation statistical analysis using Pearson's test was applied on results to find relationship between concentration of extracts and inhibition zones using SPSS software version 20.0.

RESULTS AND DISCUSSION

Spices extracts are in use as an active ingredient in many herbal medicines and are proved too have a number of pharmacological effects to treat different human diseases and also widely used to prevent diseases in fish [37, 47]. Alcohol e.g. Methanol and Ethanol extracts of herbal spices have been evaluated *in vitro* against *P. aeruginosa* as it is a well-known as causative agent of so many resistant nosocomial infections in all six plates no inhibition zone around PBS wells and discs. Nearly all extracts at their concentrations showed inhibition pattern. Among all concentrates, the higher conc. of 100mg/mL (10^{-1}) of *N. sativa* showed most remarkable antibacterial activity in both well and disc diff. methods. The inhibitory pattern of all three ethanol extracts against *P.aeruginosa* around the wells and discs shown in Table 1, Ethanol extract of *N.sativa* seeds were more effective as antibacterial against *P.aeruginosa*. The highest inhibition zone was observed with ethanol extract of 10^{-1}; the highest conc, 100mg/mL, it efficiently inhibited growth of tested bacterium. Comparison of effects of ethanol extracts of spices is shown in Fig. 1. The project under study is in agreement with report that spices extracts have antibacterial activity against wide range of microbes including *P.aeruginosa* [38]. During extraction process of spices, solvents e.g. ethanol diffuse into the solid plant material and soluble compounds of similar polarity [39]. The extraction of biologically active compounds from plant material depends upon type of solvents used in extraction procedure. The polarity of solvents affects quantity and composition of secondary metabolite of extract. Organic solvents have been found to give more consistent antibacterial activity compared to other extracts [40]. Most other bioactive compounds including phenols are generally soluble in polar solvents such as ethanol. Due to such activities phytomedicines are being used to treat diabetes, malaria etc [41, 48]. Methanol, Ethanol most commonly used solvents for antibacterial investigations [42]. One of these active ingredients is Thymoquinone (Volatile oil of *N.sativa* seeds) and fixed oil, eugenol, allicin [43]. Generally, higher levels of proteins and carbs contents of extracts have better antimicrobial activity [44]. Puroindoline is the main component of new family of proteins suggested to exert an antimicrobial activity in plant seeds and is found abundantly in wheat as in wheat growing regions like China, Middle East and India [45, 46, 49]. The study provides support to the antibacterial potential of spices extracts as herbal Ethnomedicine as a potent antimicrobial agent. On the basis of above results, it showed that ethanol extracts of spices typically *N.sativa* pronounced greater inhibitory effect compared to other extracts (Fig. 1) Statistical test Bivariate Correlation applied on results shows a strong correlation of nearly 72% between conc. of extracts and inhibition zones (Table 2).

Table 1: Inhibitory pattern of Ethanol extracts of spices

Name of Extract	Dilution	Conc. (mg/mL)	Inhibition Zones	
			Wells	Discs
Nigella sativa	10^{-1}	100	++	++
Nigella sativa	10^{-2}	10	+	+
Nigella sativa	10^{-3}	1	+	+
Allium sativum	10^{-1}	100	++	++
Allium sativum	10^{-2}	10	+	+
Allium sativum	10^{-3}	1	+	+
Syzygium aromaticum	10^{-1}	100	+	+
Syzygium aromaticum	10^{-2}	10	+	+
Syzygium aromaticum	10^{-3}	1	-	-

++= >15 diameter inhibition zone
+= ≤15 diameter inhibition zone
+= ≤07 diameter inhibition zone
-= No inhibition zone

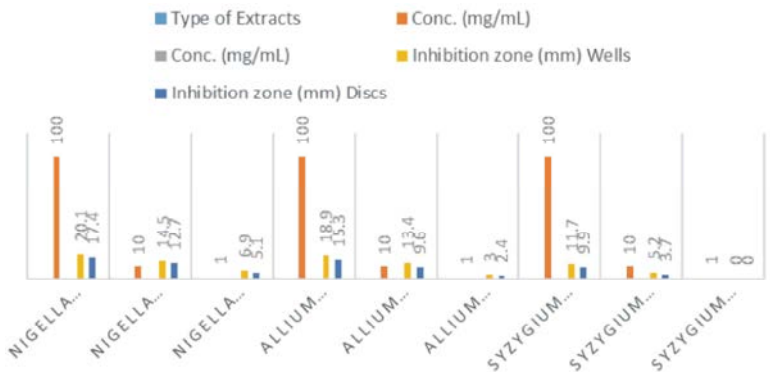

Fig. 1: Comparison of Effects of Ethanol extracts of Spices

Table 2: Correlation between Concentration of Extracts and Inhibition Zones for both wells and discs

		Conc. of Ethanol Extracts	Inhibition zone (wells)	Inhibition zone (Discs)
Conc. of extracts	Pearson Correlation	1	.728*	.749*
	Sig. (2-tailed)		.026	.020
	N	9	9	9
Wells	Pearson Correlation	.728*	1	.994**
	Sig. (2-tailed)	.026		.000
	N	9	9	9
Discs	Pearson Correlation	.749*	.994**	1
	Sig. (2-tailed)	.020	.000	
	N	9	9	9

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

CONCLUSION

It may be concluded from results of project under study that all spices extracts have well antibacterial activity against *P.aeruginosa*, due to their active bio components in combination with the alcoholic solvent ethanol. But if we observe the comparison pattern of extracts outputs reported that in contrast to others, *Nigella sativa* ethanol extract pronounced the most remarkable antibacterial activity against the tested bacterium. And the current study might suggest the use of herbs and spices extracts along with its bioactive constituents and antiseptic solvents e.g. ethanol as ethnomedicine and for so many other medical purposes depending upon the type and conc. Of extracts under application and so the infection too.

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