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RESEARCH ARTICLE

IN VITRO ANTIBACTERIAL ACTION OF NATIVE AND DOMESTIC SPICES INCLUDING BLACK CUMIN, GREEN CARDAMOM, AND CINNAMON, (NATURAL ANTIMICROBIALS)

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ABSTRACT

Many natural products including medicinal plants have been contributed in the development of drugs or antimicrobials thus spices retaining medicinal particularly antimicrobial potentials have also played a great role in this field. *Carum carvi* (black cumin), *Elettaria cardamomum* (green cardamom), and *Cinnamomum zelanium* (Cinnamon) has shown their beneficial effects against some of the clinical isolates including *Staphylococcus aureus, Enterococcus sp., Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia, Acenatobacter sp., Proteus sp.,* and *Escherichia coli* that are becoming extremely disappointed problems in present days and have failed to respond the previously active drugs. So it is important to explore various natural products to find new of this type as preventive measures. In this regard spices can be correctly said to natural antimicrobials.

INTRODUCTION

"Eat leeks in March and wild garlic in May, and all the year after the physicians may play." Traditional Welsh rhyme (Marjorie Murphy Cowan, 1999). It is a common phrase that "Enemy's enemy is a friend" therefore many natural products are our friend to fight against various diseases causing microorganism. As far as antimicrobials are concerned wild plants as well as other cultivated plants have played an important role to treat microbial infections. Even cultivated plants have more advantageous and better therapeutic substances due to greater flexibility to commercial propagation. Various parts especially essential oils from leaves and flowers of these both categories are most appreciable in this respect (Hubert A. Harris, 1949; El Bouzidi *et al.,* 2012). Spices classically or expressively can be defined as any of a class of pungent or aromatic substances of vegetable origin, used as a flavoring e.g. pepper, ginger, cinnamon, or cloves, used as seasoning, preservatives, etc (http://dictionary.reference.com).

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While these spices where having the traditional role, their role in the world of medicine is of equal importance which of course cannot be denied. Such properties that to work as antimicrobials as well as management of other medical and physiological problems including Women's personal illness (de Boer and Cotingting, 2014) they are accurately called medicinal plants. "The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities". These medicinal plants are considered as rich resources of ingredients which can be used in drug development and synthesis (Bassam Abdul Rasool Hassan, 2012). More over it has been scientifically proved that, herbs and these plants have voluminous medicinal activities such that modern pharmaceuticals have been derived from medicinal plants (Lai and Roy, 2004; de Boer and Cotingting, 2014). A number of plants are being used as cure for bacterial infections; conventional drugs provide effective treatment against bacterial infections however the growing problem of resistance, leads to continuous requirement of new solutions. Even if natural materials are not necessarily safer but still some people prefer to use herbal medicine (Martin and Ernst, 2003). Microbial infections are a great health problem throughout the World, and plants are a feasible source of antimicrobial agents

because they possess active principles which can be used as an alternative to cheap and effective herbal drugs against common bacterial infections (Kareru *et al.,* 2007). Medicinal plants are one of the important needs of men. The importance of medicinal plants becomes more obvious at the present time in developing countries. In Pakistan it is estimated that 80% of its population depends on plants to cure them selves, 40% in China. In technological advanced countries such as United States, it is estimated that 60% of its population use medicinal plants habitually to fight certain ailments. In japan there is more demand of medicinal plants than of official medicines (www.botanical-online.com/english.htm). Pharmaceuticals became popular in Europe during 19th century after development in chemical analysis techniques that allowed scientists to isolate and extract beneficial plant constituents. Before this scientific investigation system, traditional use of medicinal plants involved utilizing an entire portion of the plant like the root or leaf, rather than extracting a single component. Pharmaceuticals also go through extensive testing before being available to the public, although scientists report more side effects with drugs than medicinal plants (http://www.ehow.com). Ayurveda is one the traditional medicine system widespread in India and other South Asian countries and medicinal plants including herbs especially of Indian origin, are the essence of the system (Parasuraman *et al.,* 2014). Table 1 summarizes different aspects of some of the spices used for flavoring and pleasant smell.

Table 1. Importance of Spices

Spices	Common Names	Family	Short Description	Parts used	Traditional Use
Carum carvi	Caraway	*Apiaceae* or *Umbelliferae* (http://www.seedaholic.com/caraway-carum-carvi.html, http://www.holisticonline.com/herbal-med/_Herbs/h117.htm)	Biennial, valuable aromatic herb (Chizzola, 2014), excellent source of minerals (http://www.nutrition-and-you.com/ caraway-seed.html),	Fruit (seeds).	Gastric problems, eye infections, toothaches and rheumatism (http://health-from-nature.net/ Caraway.html). Food preservative (Lixandru *et al.,* 2010)
Elettaria cardamomum	Cardamom small.	*Zingiberaceae* (http://www.iloveindia.com/indian-herbs/cardamom.html, http://www.holisticonline.com/herbal-med/_Herbs/h117.htm)	Evergreen, perennial ginger-like plant, sweeten the breath and to detoxify caffeine (http://www.iloveindia.com/ indian-herbs/cardamom.html), "Queen of Spices" (Sharma, 2012), reduces toxic effect of pan masala (Kumari and Dutta, 2014).	Seed.	Aromatherapy, Ayurvedic, Bronchitis, Congestion, Digestion, Herbal Steam Herbal Teas, Nausea (http://www.anniesremedy.com/herb_detail11.php).
Cinnamomum zelanicum	Cinnamon.	*Lauraceae* (http://www.anniesremedy.com/ herb_detail15.php)	commonly planted for ornamental purposes (http://www.tradewindsfruit.com/ content/cinnamon.htm), therapeutic agent for diabetes (Ranasinghe *et al.,* 2012)	Dried bark, Essential oil from leaves (http://www.anniesremedy.com/ herb_detail15.php http://herbalhealing. tumblr.com/post /14343454609/cinnamon-cinnamomum-zeylanicum-parts-used-bark, http://www.essentialoils.co.za/essential-oils/ cinnamon-leaf.htm).	Remedy for respiratory, digestive and gynecological ailments (Ranasinghe *et al.,* 2012; Priyanga Ranasinghe *et al.,* 2013).

Table 1 shows various aspects of subjected spices including precise information, medicinal importance, etc.

MATERIALS AND METHODS

Bacterial species to be tested

In the present study clinical isolates including *Staphylococcus aureus, Enterococcus sp., Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia, Acenatobacter sp., Proteus sp.,* and *Escherichia coli* were used. All Organisms were sub cultured and maintained on nutrient agar (Oxoid) plates and slants at 37°c.

Microbiological Media

Microbiological Media including Nutrient agar, MHA, and Nutrient broth (Oxoid) were used.

Chemicals

DMSO (Dimethyl sulfoxide), McFarland index 0.5, and Normal Saline (for bacterial culture suspension).

Spices purchasing and validation

All spices were purchased from local markets and identified then. Their voucher depositing numbers were deposited in the department of pharmacognosy and then their extracts were provided by Dr. Iqbal Azhar (Professor, Pharmacy Department University of Karachi).

Extracts preparation

The samples (spices) were soaked in ethanol separately at room temperature for 15 days. All these were filtered and then evaporated by using rotary evaporator finally semidried extracts were obtained.

Extract solution

As the extracts were semidried so that, they were dissolved into DMSO to use them in convenient form. For this purpose 0.5g was dissolved in 10 ml.

Antimicrobial for comparison

Berberis vulgaris.

Antimicrobial Examination

To check *in vitro* antimicrobial activity assays includingAgar well diffusion test (Holder and Boyce, 1994) and Micro titer broth dilution method (Paul K Lunga *et al.,* 2014) were performed.

Agar Well Diffusion Assay

PRINCIPLE: The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters (http://shodhganga.inflibnet.ac.in/ bitstream/10603/1458/11/11_chapter3.pdf). Petri plates containing 25-30ml Muller Hinton medium were seeded with 24hr culture of bacterial strains after solidification. Suspensions of the test organisms were made in normal saline and 10^8cells/ml were maintained by using McFarland index 0.5. Wells were prepared by using alcohol dipped sterile borer of 7mm in diameter by maintaining suitable space between each well. 100 µL (5% w/v) of plant extracts were added with the help of micropipette in different wells in the plate and left it at room temperature for about 30 min. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well and expressed in mm as its antimicrobial activity. Each test was run in triplicate and repeat (n=6).

Determination of MICs by Micro titer broth dilution/ Micro Dilution method

PRINCIPLE: Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial needed to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in log2 serial dilutions (two fold) (http://shodhganga.inflibnet.ac.in/bitstream/10603/1458/11/11_ chapter3.pdf). The selected bacterial strains were inoculated onto nutrient agar slants to grow overnight. Overnight cultures were suspended in sterile normal saline and 10^8cells/ml was maintained by using McFarland index 0.5. This is the bacterial sample to be evaluated for antimicrobial sensitivity or resistance. 100ul of Muller Hinton broth was dispensed into all wells of a microtiter plate. The plate was labeled, as each row for a particular bacterial culture. 100ul of antimicrobial was pipetted into the wells in column 1 (far left of plate). Using the multi pipettor set at 100ul, the antimicrobial was mixed into the wells in column 1 by sucking up and down at least 3-4 times. Do not splash. 100ul was withdrawn from column 1 and added to column 2. This makes column 2 a twofold dilution of column 1. After mixing up and down 3-4 times 100ul was transferred to column 3. The procedure was repeated down to column 11 only. The same set of tips can be used for the entire dilution series. 100ul was discarded from column 11 rather than putting it in column 12. With the smaller multipipettor set to 10ul, bacterial culture suspension was dispensed into all wells except well 11, thus well 11 contained medium and plant extract only thus acting as sterility control and blank for the plate reader, containing only medium and antimicrobial. Bacterial culture suspension was added to column 12 i.e. column 12 contains medium and bacterial culture only for comparison. This protocol was followed for all the selected antimicrobials in separate microtiter plates. All the plates were incubated at 37oC for 24 hours. The plates were scanned with an ELISA reader (Magellan software). MIC of each extract was taken as the lowest concentration or highest dilution that did not give any visible bacterial growth or the lowest concentration of extract that reduces, by more than 50% or 90% for MIC_{50} or MIC_{90} respectively. At this concentration or dilution extracts were bacteriostatic.

Time kill Assay

Grow culture of *E. coli* (over night) in nutrient broth. Next day culture was matched with Macfarlane index to give rise to 108 cells per ml. 10ml sterile nutrient broth was taken in test tube and only 10µl was discarded then 10µl culture was added that diluted it to 10-3 or 1: 1000. At last 1ml diluted culture and 0.1ml extract was transferred into a well dried and clean cuvettes finally readings for absorbance were taken at 600 nm using spectrophotometer at 0minutes, 30 minutes, 60 minutes, 120 minutes, 240 minutes and 1440 minutes. Similar procedure was followed for all the extracts.

RESULTS

Antimicrobial activity in terms of their susceptibility or resistance to the tested antimicrobial (spices samples) is given in Table 2.

Table 2. Zones of inhibition against spices' Ethanol extracts

Organisms	Zone of Inhibition (avg) using Agar Well Diffusion Method (mm)			
	Carum carvi	*Elettaria cardamomum*	*Cinnamomum zelanium*	*Berberis vulgaris*
Staphylococcus aureus	13.5	12	10	21
Enterococcus feacalis	ND	10	ND	16
Pseudomonas aeruginosa	10	ND	13	19
Klebsiella pneumonia	12.5	13.6	11	11
Salmonella typhi.	13	12	12	15
Escherichia coli	11.8	10	14	15
Acenatobacter sp.	11.6	12	14	10
Proteus sp.	08	ND	10	11

Table 1 shows average of triplicate and repeat inhibition zones of eight clinical isolates against extracts of spices in comparison to *Berberis vulgaris* obtained in Agar Well Diffusion Method. ND: Not Determined

Table 2 summarizes the sensitivities of ethanol extracts of spices against*Staphylococcus aureus, Enterococcus feacalis, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Escherichia coli, Acenatobacter sp.,* and *Proteus sp.* All

the test organisms appear to be sensitive to spices' extracts(inhibition zone diameter 08 – 14 mm), except *Enterococcus feacalis* that shows no zone in response to extracts of *Carum carvi*and *Cinnamomum zelanium*similarly *Pseudomonas aeruginosa* and *Proteus sp.*were not showing sensitivity to*Elettaria cardamomum*. Among all the samples tested, *Carum carvi*gave the smallest inhibition diameter against *Proteus sp.* (8 mm). Standard antimicrobial *Berberis vulgaris* gave zones of inhibition of 10-21 mm, which were higher or comparable to those of the tested extracts. T – Test comparison of *Carum carvi, Elettaria cardamomum,* and *Cinnamomum zelanium* separately with standard shows (p=0.05, =0.05, and >0.05) respectively. For further confirmation of the results and to know the exact concentration of the sample to be antimicrobial micro titer broth dilution method was performed. Results of the assay are shown in Table 3.

Table 3. MIC of Spices' Ethanol extracts

Organisms	MIC(mg/ml)			
	Carum carvi	*Elettaria cardamomum*	*Cinnamomum zelanium*	*Berberis vulgaris*
E. coli	0.39	0.78	6.25	3.125
E. feacalis	0.195	6.25	3.125	6.25
Klebsiellapneumoniae	0.39	0.195	3.125	1.56
Salmonella typhi	0.39	6.25	1.56	6.25
Pseudomonas aeruginosa	0.39	1.56	1.56	6.25
Acenatobacter sp.	0.39	3.125	3.125	3.125
S. aureus	0.39	6.25	1.56	25
Proteus sp.	0.195	3.125	6.25	12.5

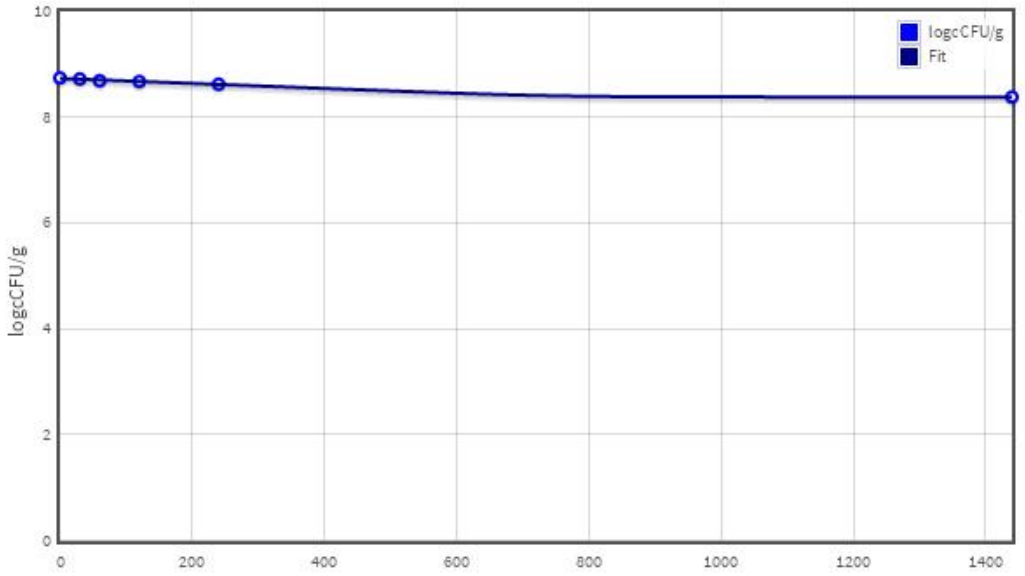
Table 2 shows minimum inhibitory concentration of the extracts of spicesin comparison to *Berberis vulgaris* against eight clinical isolates determined by Micro titer broth dilution method

The above results explain that extracts of the test spices are very effective in very less concentration that ranges from 0.195 to 0.39, 0.195 to 6.25, and 1.56 to 6.25 mg/ml, in case of *Carum carvi, Elettaria cardamomum,* and *Cinnamomum zelanium*r espectively against all of the tested bacterial species. P values from T. TEST for each sample in comparison to *Berberis vulgaris* are <0.05, >0.05, and >0.05 respectively.

Finally time killing assay was performed against *E. coli* to examine the efficacy of each extract. Following figures depict the efficiency of the sample extract that at which exact time sample starts to work and then eventually its activity drops.

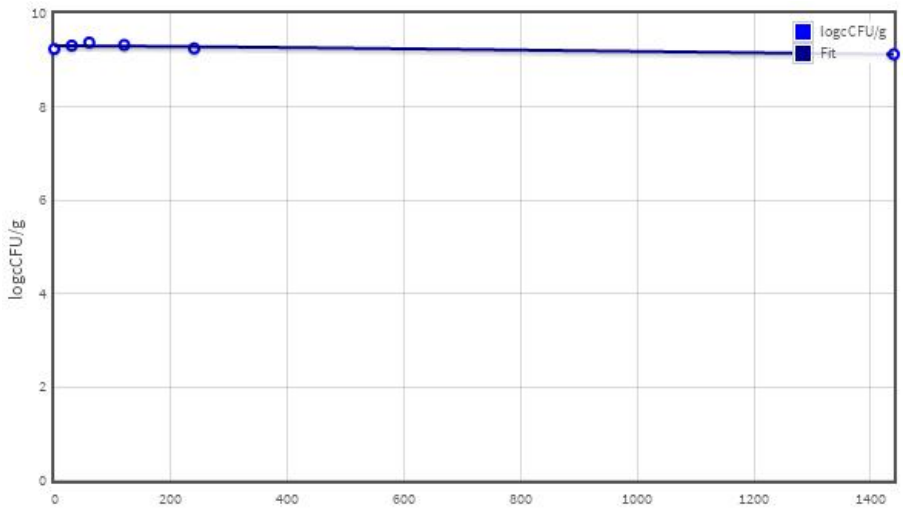
These figures depict the exact time of the activity of the antimicrobials in question, Figure 1 depicts the activity ofstandard antimicrobial *Berberis vulgaris*that microbial load gradually decreases from 0 to 1440 minutes.

Carum carvi on the other hand began to work one hour after exposure as shown in Figure 2 gradual increase in log cfu/ml even after exposure to extract after 60 minutes log cfu/ml decreases from 9.38 to 9.15 within 24 hoursthus it retains its activity by 24 hours, *Elettaria cardamomum*showing drastic decrease after exposure (Figure 3) and *Cinnamomum zelanium* (Figure 4) started to work after exposure but lost its activity at 30 minutes thus as soon as it started to work gradually increase in log as not all the cells might be killed at a time some of them survive and take more time to be killed. T. TEST for comparison of all three spices extracts with standard shows *p value (<<0.05, >0.05 and <<0.05)* respectively.



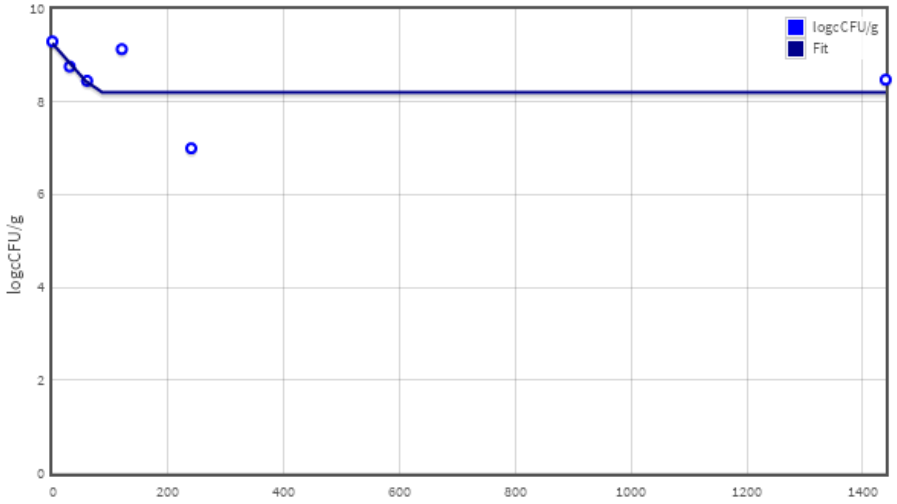
Key: x-axis= Time in minutes, y-axis=log CFU/ml; extent of bacterial reductions as indicated by the Log (cfu/mL) atthe respective sampling times. Web edition (Dynamic modeling fit). R^2 = 0.993 and SE of Fit = 0.0114.

Figure 1. In vitro time-kill assessment of *Berberis vulgaris* fitted in DMFit



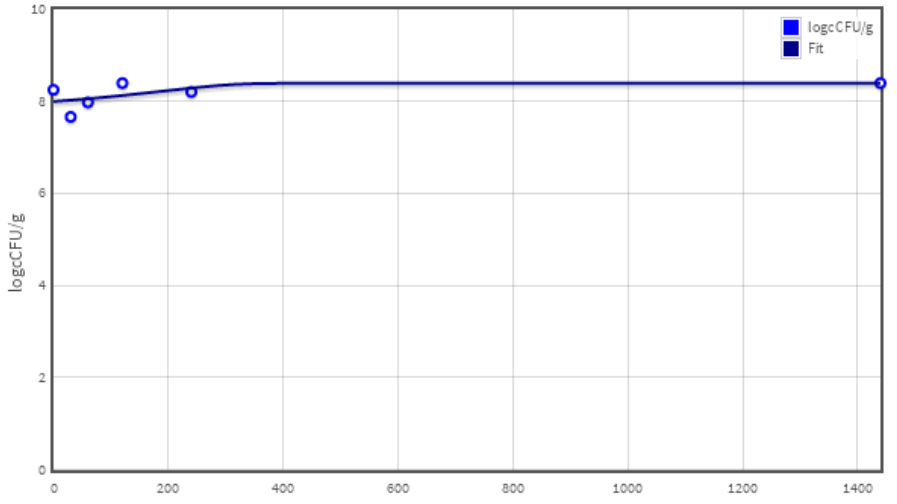
Key: x-axis= Time in minutes, y-axis=log CFU/ml; extent of bacterial reductions as indicated by the Log (cfu/mL) at the respective sampling times. Web edition (Dynamic modeling fit). R^2 = 0.462 and SE of Fit = 0.0646.

Figure 2. In vitro time-kill assessment of *Carum carvi* fitted in DMFit



Key: x-axis= Time in minutes, y-axis=log CFU/ml; extent of bacterial reductions as indicated by the Log (cfu/mL) at the respective sampling times. Web edition (Dynamic modeling fit). R2 = - 0.189 and SE of Fit = 0.895.

Figure 3. In vitro time-kill assessment of *Elettaria cardamomum* fitted in DMFit



Key: x-axis= Time in minutes, y-axis=log CFU/ml; extent of bacterial reductions as indicated by the Log (cfu/mL) at the respective sampling times. Web edition (Dynamic modeling fit). R2 = - 0.706 and SE of Fit = 0.365

Figure 4. In vitro time-kill assessment of *Cinnamomum zelanium* fitted in DMFit

DISCUSSION

Tips or advices for the treatment of any medical problem often involves the use of kitchen spices that add flavor and pleasant smell to our traditional cuisine, but the prescription of these spices have scientific background which can be better understood by the above displayed results such that the selected spices for antimicrobial activity have been greatly proved to be antimicrobial agents. The above tables and figures depict that *Carum carvi, Elettaria cardamomum,* and *Cinnamomum zelanium*have potential to be used as initial material for new drugs. Bacterial species for which results were not determined in agar well diffusion assay appeared to be inhibited by less concentration of the samples which may be due to several reasons that could not reveal sensitivity in diffusion assay; reasons may include large inoculum on agar plate or over growth of the test bacterial isolates. Killing assay shows that the *Carum carvi* and *Elettaria cardamomum,* are very effective against *E. coli*. Finally the obtained results give evidence to use domestic spices in designing new and potent antibacterial drugs, as emergence of resistance is great problem now a days in the field of medicines which has led to failure in the effective treatment of various infections. Even we use such spices in our daily lives as our traditional tips or advices without knowing their medicinal especially antimicrobial activity which they actually possess. Variation in the results might be due to adulteration in the original pure spices as they were purchased from local market.

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