

Individual and Combined Antibacterial Activity of plant Essential Oils and Antibiotics Against Bacterial Isolates of Mastitis

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ABSTRACT

The current study aims to investigate the individual and combined antimicrobial efficacy of antibiotics and some plant essential oils (EOs) viz., tea tree, lavender, black seed, and clove against mastitis causing common bacterial pathogens, i.e., *Streptococcus spp.*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using disc diffusion method. The black seed produced significantly higher ($p < 0.05$) inhibitory effects against *Pseudomonas aeruginosa* as compared to tea tree, lavender, and antibiotics ampicillin and neomycin. Clove produced significantly

higher ($p < 0.05$) inhibitory effects against all tested bacteria as compared to lavender and tea tree. Black seed and clove produced significantly higher ($p < 0.05$) inhibitory effects against *Escherichia coli* as compared to tea tree, lavender, and antibiotic ampicillin. Similarly, tea tree produced significantly higher ($p < 0.05$) inhibitory effects against *Escherichia coli* as compared to lavender and antibiotic ampicillin. The clove and lavender when combined with neomycin, and black seed with oxytetracycline exhibited the synergistic antimicrobial effects against *Staphylococcus aureus* and *Streptococcus spp.* Similarly, clove+neomycin, clove+ampicillin, lavender+neomycin, tea tree+ampicillin, and black seed+oxytetracycline produced

the synergistic antimicrobial effects against *Escherichia coli*. Likewise, the combinations of clove+neomycin, clove+oxytetracycline, lavender+neomycin, tea tree+neomycin, and black seed+oxytetracycline produced the synergistic antimicrobial effects against *Pseudomonas aeruginosa*, whereas all other combination of EOs and antibiotics showed antagonistic effects. The study concluded that EOs have significant antimicrobial potential both individually and in combinations with antibiotics against mastitis isolates.

INTRODUCTION

Mastitis in bovine is a mammary gland infection and an important problem for lactating animals that lead towards decrease in milk synthesis, and frequently related with cattle disorders such as rumen motility and fever.¹ Massive number of commercially available antimicrobial agents cause drug resistance, changes in enteric microbiota, super infections, and several other negative effects because of the increase in chemo resistance of few bacterial strains.² The treatment of mastitis with antimicrobial drugs is most commonly based on intramammary infusion. Furthermore, a misuse or a utilization of untargeted antimicrobial agents could lead to severe concerns for health of the public. In recent strategies, the World Health Organization ensured to limit the usage of antibiotics in livestock, particularly in food animals.³

Antibiotic resistance is phenomenon as old as the advent of antibiotics. Antibiotics are produced from natural substances like fungi and also by certain bacteria to defend against other bacteria.⁴ The development and spread of resistance to present available antibiotics is a worldwide concern.⁵ The increase of resistance problem in human and veterinary medicine requires to go for new therapeutic choices and to determine the antimicrobial properties of the medicinal plants and or their bioactive molecules, including flavonoids, phenolic compounds, resins, tannins, steroids, fatty acids, and gums that are found in plant Eos.⁶

The Eos, unlike antibiotics, are made up of many molecules so that bacteria cannot resist in mutant.⁷ Curatively and preventively, they are mainly known for their potent antiviral, antimicrobial, antifungal, antiparasitic, expectorant, mucolytic, antipyretic, and anti-inflammatory effects. The combination of antibiotics with essential oils may lead towards new way to cure infectious diseases with extended degree of efficacy.⁷ As the other studies reported that black seed oil (*Nigiva sativa*) have strong antibacterial effects against the *Listeria monocytogenes* bacteria and showed a greater zone of inhibition than that of standard antibiotics gentamicin.⁸ Clove oil also been reported as a most powerful agent against the periodontopathic microbes including *Prevotella intermedia*, *Fusobacterium nucleatum*, *Pophyromonas gingivalis*, *Prevotella melaninogenica*, *Capnocytophaga, gingivalis* and *Actinobacillus actinomycetemcomitans*, and some superinfectant pathogens like *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, and *E.coli*.⁹

In the light of above literature showing gravity of antimicrobial resistant issues and potential of EOs against bacterial pathogens, the present research was conducted to evaluate the in vitro antibacterial activity of plant essential oils (viz., clove, lavender, tea tree, and black seed) against mastitis bacterial isolates by disc diffusion method. The study also demonstrated the combined efficacy of EOs with standard antibiotics against mastitis isolates.

MATERIALS AND METHODS

The bacterial organisms viz., *Streptococcus spp.*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were isolated and identified from mastitis buffalo milk samples. Milk samples (n= 20) were collected from those animals that have frequent incidences of mastitis and currently had mastitis from last ≥ 5 days, and showed poor response to antibiotic treatment. The isolates from such milk samples were as-

Table 1. Individual antimicrobial activity of essential oils and antibiotics against mastitis isolates.

Name of the Agents*	Inhibition zone diameter (mm)			
	<i>Staphylococcus aureus</i>	<i>Streptococcus spp.</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Clove	8.6#	9.3#§	5.2#§^	2§
Lavender	7.5#	7#	2	0.5
Tea tree	4.5	3.7	4§^	1.7§
Black seed	10#§	10#§	6#§^	11.7#§^~
NOR	20.1	19	12	12.5
OT	17.5	17	18	17.2
AMP	10.4	10	1	2.5
N	10.5	10.6	9.6	9

* NOR: norfloxacin, OT: oxytetracycline, AMP: ampicillin, N: neomycin

Significantly higher than Tea tree at $p < 0.05$

§ Significantly higher than Lavender at $p < 0.05$

^ Significantly higher than reference antibiotic Ampicillin at $p < 0.05$

~ Significantly higher than reference antibiotic Neomycin at $p < 0.05$

sumed as resistant in terms of antimicrobial therapy. Four essential oils viz., tea tree (*Melaleuca alternifolia*), lavender (*Lavandula angustifolia*), black seed (*Nigella sativa*), and clove (*Syzygium aromaticum*) were purchased from local market. These were evaluated individually and in combination with antibiotics, for their antibacterial activity against bacterial organisms isolated from bovine mastitis milk samples.

Isolation and Identification of Mastitis Isolates

Isolation of mastitis causing bacterial organisms was done by using conventional culture procedure as described previously by.¹⁰ A total of 15 isolates were recovered for each bacterial specie. In brief, mastitis milk samples were cultured on different culture media using sterile wire loop and then streaked agar plates were incubated overnight at 37°C. Then selected bacterial colonies were picked up with the help of sterile loop from cultured agar plates and transferred to separate media plates or culture tubes to form a pure bacterial culture. The species of isolates were confirmed using standard identification procedures.¹¹

Antibacterial Activity of Essential Oils

(EOs)

Antibacterial activity of essential oils was determined by disc diffusion method following the procedures of Clinical and Laboratory Standard Institute (CLSI).¹² Shortly, Mueller Hinton media was prepared. Microorganisms cultures having optical density equal to 0.5 McFarland units were spread over agar plates (media surface). The sterile paper discs (6 mm diameter) were impregnated with 2µl each of pure essential oil and were placed on the inoculated agar surface. Standard antibiotic discs (viz., norfloxacin, oxytetracycline, ampicillin, and neomycin (Oxoid, UK) were used to compare the zone of inhibition with EOs. Plates were incubated at 37°C for 24 hrs, then activity was determined by measuring diameter of zone of inhibitions. All tests were performed in triplicate.¹³ To determine the combined effect of EOs with antibiotics 2µl of each EOs was saturated with antibiotic disc to determine the zone of inhibition.¹⁴ The obtained results were compared with those of the antibiotics and essential oils tested on the same bacteria alone.

Statistical Analysis

Mean inhibitory zones of EOs and anti-

Table 2. Combined antimicrobial activity of essential oils and antibiotics against mastitis isolates.

Name of the Agents*	Inhibition zone diameter (mm)#			
	<i>Staphylococcus aureus</i>	<i>Streptococcus spp.</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Clove + NOR	20.5 (A)	20.4 (A)	10.7 (A)	10.7 (A)
Clove + N	20.3 (S)	20.3 (S)	15.3 (S)	12.4 (S)
Clove + AMP	10 (A)	10 (A)	10.3 (S)	3.5 (A)
Clove + OT	10.5 (A)	10.4 (A)	20.4 (A)	20.2 (S)
Lavender + NOR	20.3 (A)	20.1 (A)	16.4 (A)	10.7 (A)
Lavender + N	20.7 (S)	20.8 (S)	12.4 (S)	10.7 (S)
Lavender + AMP	10.2 (A)	10.3 (A)	2 (A)	2 (A)
Lavender + OT	9 (A)	9 (A)	10.6 (A)	10.7 (A)
Tea tree + NOR	20 (A)	20 (A)	10.5 (A)	13.5 (A)
Tea tree + N	10.4 (A)	10.5 (A)	10.6 (A)	11.6 (S)
Tea tree + AMP	10.8 (A)	10.9 (A)	10.6 (S)	3.2 (A)
Tea tree + OT	9 (A)	10 (A)	20.8 (A)	17.6 (A)
Black seed + NOR	10.9 (A)	10.8 (A)	13.8 (A)	20.4 (A)
Black seed + N	10.8 (A)	10.8 (A)	10.4 (A)	10.3 (A)
Black seed + AMP	10.5 (A)	10.9 (A)	3 (A)	3 (A)
Black seed + OT	27.5 (S)	27.8 (S)	24.6 (S)	29.1 (S)

* NOR: norfloxacin, AMP: ampicillin, N: neomycin, OT: oxytetracycline

values in parentheses showing combined effect in terms of Additive (AD), Synergism (S) or Antagonism (A); that was calculated by comparing the combined antimicrobial activity with sum of individual antimicrobial activity of corresponding essential oil and antibiotic.

biotics were compared by using one way ANOVA, which followed by Duncan's Multiple Range post hoc test using SPSS version 16.0.

RESULTS

Individual Antimicrobial Activity of Essential Oils and Antibiotics Against Mastitis Isolates

The results presented in table 1 showing the individual inhibitory activity of essential oils and antibiotics against the bacteria *Staphylococcus aureus*, *streptococcus spp.*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The data showed that the black seed produced significantly higher ($p<0.05$) inhibitory effects against *Staphylococcus aureus* as compared to lavender and tea tree. While clove and lavender also produced significantly higher ($p<0.05$) inhibitory

effects against *staphylococcus aureus* as compared to tea tree. Black seed and clove produced significantly higher ($p<0.05$) inhibitory effects against *streptococcus spp.* when compared with tea tree and lavender. Whereas, lavender also produced significantly higher ($p<0.05$) inhibitory effects against *streptococcus spp.* as compared to tea tree. Black seed and clove produced significantly higher ($p<0.05$) inhibitory effects against *Escherichia coli* as compared to tea tree, lavender, and antibiotic ampicillin. Tea tree produced significantly higher ($p<0.05$) inhibitory effects against *Escherichia coli* as compared to lavender and antibiotic ampicillin. Black seed produced significantly higher ($p<0.05$) inhibitory effects against *Pseudomonas aeruginosa* as compared to tea tree, lavender, and antibiotics ampicillin and neomycin. Clove and tea tree produced

significantly higher ($p < 0.05$) inhibitory effects against *Pseudomonas aeruginosa* as compared to lavender.

Combined Antimicrobial Activity of Essential Oils and Antibiotics Against Mastitis Isolates

Table 2 showed that the clove and lavender when combined with neomycin, and black seed with oxytetracycline exhibited the synergistic antimicrobial effects against *Staphylococcus aureus* and *Streptococcus spp.*, whereas all other combinations of EOs and antibiotics exhibited the antagonistic effects. Similarly, clove+neomycin, clove+ampicillin, lavender+neomycin, tea tree+ ampicillin, and black seed+oxytetracycline produced the synergistic antimicrobial effects against *Escherichia coli*, whereas all other combinations of EOs and antibiotics exhibited the antagonistic effects. Likewise, the combinations of clove+neomycin, clove+oxytetracycline, lavender+neomycin, tea tree+neomycin, and black seed+oxytetracycline produced the synergistic antimicrobial effects against *Pseudomonas aeruginosa* whereas all other combinations of EOs and antibiotics exhibited the antagonistic effects against *Pseudomonas aeruginosa*.

DISCUSSION

The constant developments of bacterial resistance to currently available antibiotics are increasing problems. Drug-resistant bacteria create additional cases of longer recuperation times, illness, production losses, and unnecessary deaths that needed the search for innovative and active antimicrobial compounds.^{15,16} This condition has forced scientists to examine for new antimicrobial constituents from several plants, which are the good sources of new antimicrobial chemotherapeutic agents.^{17,18} Consequently, it is essential to build up harmless and natural alternative methods for controlling the infections. Medicinal plants are known as chief sources of new chemical substances with potential medicinal effects.¹⁹ Aromatic and therapeutic plants are commonly recognized to have antibacterial effect against various

harmful disease causing agents.²⁰ Alternative cure for mastitis in bovine was carried out in recent studies with natural extracts from plants that exhibited remarkable results.^{20,21}

In the present study, black seed essential oil showed high antimicrobial effects against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus spp.*, and *Pseudomonas aeruginosa*. As the other studies reported that black seed (*Nigella sativa*) has gaining a superior attention as medicinal plant and there were several studies had reported its antimicrobial effects and proposed it as a best candidate of future antimicrobials.²² Another study showed that black seed essential oil have strong inhibitory effects against multidrug resistant *Staphylococcus aureus*.²³

Clove was recognized as a second most effective antibacterial substance against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus spp.*, and *Pseudomonas aeruginosa* in current study. Clove oil contains high amount of eugenol, that have been reported as a potential compound to inhibit the growth of Gram-negative and Gram-positive bacteria.²⁴ Clove oil also been reported as a most powerful agent against the periodontopathic microbes including *Prevotella intermedia*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *Capnocytophaga gingivalis*, and *Actinobacillus actinomycetemcomitans*, as well as some superinfectant pathogens like *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, and *E.coli*.⁹

In this study, clove and lavender when combined with neomycin and black seed combined with oxytetracycline produced antimicrobial synergisms against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus spp.*, and *Pseudomonas aeruginosa*. As explained earlier that combined activities of antimicrobial drugs may be due to certain complex formulation achieved after combination than individual drugs. This results comparatively high degree of inhibition of microbes activity.

The combined antimicrobial action of antimicrobials and essential oils are one of the modern methods to overcome multi drug resistant bacteria. Such synergistic treatments might lead towards decrease of the active dose of antibacterial agents required for therapy and also have less side effects.^{25,26} Earlier studies have declared that bacteria isolated from various animal species have varies level of antimicrobial susceptibilities that is probably because of complex host-microbe interaction that play role in the development of antimicrobial resistance.²⁷

CONCLUSION

From the results it could be concluded that essential oils (clove, lavender, tea tree and black seed) have antibacterial activity against mastitis-causing bacteria including *Streptococcus spp.*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Individually, black seed and clove have better antimicrobial effects as compared to tea tree and lavender essential oils. In combinations, clove + neomycin, black seed + oxytetracycline, and lavender + neomycin have superior antimicrobial effects and synergisms against all isolates of mastitis as compared to other combinations of EOs and antibiotics.

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