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RESEARCH ARTICLE

ANTIBACTERIAL MODE OF ACTION OF THE ROSEMARY/EUCALYPTUS OIL COMBINATION ON THE MEMBRANE INTEGRITY OF SELECTED FOOD BORNE PATHOGENS.

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Abstract

Deterioration of food can be caused by various factors such as physical, chemical, enzymatic or microbiological. Due to increasing side effect of chemicals in food, consumers demand for some natural alternative to these chemical preservatives. One of these alternatives is use of plant essential oils. The aim of this study was to understand the effect of combination of two commercial essential oils (rosemary/eucalyptus oil) on the membrane of one gram positive and one gram negative bacteria at MIC concentration. The release of cell constituents at 260 nm and potassium ions from the bacterial cell were measured after they were treated with essential oil combination of rosemary /eucalyptus oil at their MIC concentrations. The result showed that there was damage in the cell covering of bacteria because of the treatment of combination of oils. The assays performed to study the effect of combination of oil on cell membrane of both selected bacteria exhibited that the combination at MIC concentration damaged the cell membrane of both bacteria. These results provide the useful information that the rosemary /eucalyptus oil combination might be used as efficient and safe natural antimicrobial agent in food industry.

Introduction:-

Essential oils are the aromatic liquids, result of secondary metabolism in plants (**Guenther, 1948**). Essential oils are natural antimicrobials, which are used in flavor and fragrance industries **(Van de Braak and Leijten, 1999)**. In food industries the essential oils are used as natural preservatives, which are the demand of time. As the chemical preservatives have their own set of problems like carcinogenicity, teratogenicity, acute toxicity and the environmental problems they cause due to long degradation time taken by them (**Smid and Gorris, 1999**).

Essentials oils have antibacterial, antifungal, antioxidant and anticarcinogenic properties which help them to be used as food additives. Essential oils when used as antimicrobial in food are required in higher quantity, which can demonstrate negative effect in taste and odor of food (**Yamazaki et al., 2004.)**. To tackle this problem the combinations of different oil can be used which solves two purposes, one being the increase in antimicrobial effect and second is the decrease of concentration required of the oils.

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Rosemary and Eucalyptus oils are good antimicrobials alone. There is no information regarding their antibacterial action on bacteria. Hence, in the present study the mode of action of this combination at MIC value for *E. coli* and *Micrococcus yunnanensis* will be studied on their cell walls by determining the release of cellular materials at 260 nm and potassium ion efflux.

Materials and Methods:-

Material:-

Essential oil combination:- Rosemary/Eucalyptus oil combination.

Bacterial strains:- *Micrococcus yunnanensis, E. coli.*

Methods:-

Preparation of inoculum:- The bacterial inoculum used for testing the activity was prepared in nutrient broth. Five ml of broth was suspended in test tubes and autoclaved. The medium was cooled and inoculated with a loopful of bacterial cultures from the nutrient agar slants under aseptic conditions and then incubated at 37±1^{o}C for 24 hours. In order to standardize the inoculums density for test, $BaSo_4$ turbidity standard equivalent to 0.5 McFarland (turbidity equals to 0.5= 1 to 2×10^8 CFU /ml) was used (**Burt, 2004**).

Determination of minimal inhibitory concentration (MIC):- Investigation of the MIC of Rosemary and Eucalyptus essential oils was done in 96 well titre plate (**Schelz *et al.*, 2006)**. In each well Muller Hinton broth (90 µl) was added. The essential oils alone were taken from range100 µl to 3.25 µl along x axis. 10 µl of working inoculum suspension (8×10^8 CFU/ml) was added to the each well. The bacteria tested were 80 µl in which well. Ciprofloxacin was used as positive control and DMSO was used as negative control. The plate was incubated at 37^{o}C for 24 hours. The plates were then incubated for 24 hour at 37°C. After incubation, O.D was measured using ELISA reader at 595 nm. The lowest dilution showing no visible growth was considered as the MIC for that individual oil. The tests were performed in triplicate.

Determination of Fractional Inhibitory Concentration Index (FICI):- Fractional inhibitory concentration index was determined by checkerboard titration method but with some modifications (**CLSI, 2005)**. Along the rows Rosemary oil concentration was reduced to half in each subsequent well, whereas Eucalyptus oil concentration was fixed at 50 µl and in another row eucalyptus oil concentration was reduced and rosemary oil concentration was fixed at 50 µl. The bacteria tested were 80 µl in which well. Ciprofloxacin was used as positive control and DMSO was used as negative control. The plates were then incubated for 24 hour at 37°C. After incubation, O.D was measured at 595 nm. The combination which showed maximum inhibition at lower concentration was taken as MIC.

Fractional inhibitory concentration indices (FICI) were calculated using the formula:

FICI = (MIC of EOA in combination with EOB / MIC of EOA alone) + (MIC of EOB in combination with EOA/ MIC of EOB alone).

Where, EOA= rosemary oil and EOB= eucalyptus oil. The results were interpreted according to FIC indices as follows:

FICI ≤0.5: Synergy; **0.5** < FICI ≤ **4**: Additive; and FICI > **4**: Antagonistic (**Leclercq *et al.*, 1991**). All the experiments were repeated thrice.

Mode of action:- The effect of combination of essential oils at its MIC concentration was studied on cell membrane of both selected bacteria by following assays:

Potassium leakage assay:- The potassium leakage from cells was studied by following the methodology of **Cox *et al.* (2000)**. 2 ml Suspensions of selected bacteria were exposed to essential oils combination (MIC) in sterile peptone water (0.1 g/ 100mL) for 0, 30, 60, and 120 minutes at 37^{o}C. After predetermined interval the samples were collected and extracellular potassium concentration was measured by photometric method. Controls were devoid of Essential oils combination. Results were calculated as amount of extracellular potassium (mmol/L) in the growth media at each interval of time.

Leakage of 260 nm and 280 nm cellular materials:- This assay was performed according to the methodology of **Carson *et al.* (2002).** To 2 ml of bacterial culture in sterile peptone water (0.1 g/ ml) essential oil(s) combination at their MIC concentration was added. The mixture was incubated at 37 ^{o} C for 0, 30, 60 and 120 minutes. Cells for measurement were collected after 0, 30, 60, and 120 minutes, cells were then centrifuged at 3500 rpm for 15

minutes. The supernatant were collected and absorbance was measured at 260 nm UV/Vis Spectrophotometer. Controls were kept under same conditions, except that they were not treated with essential oils combination. The experiment was performed in triplicates and mean values were plotted against time.

Results:-

Determination of Minimum Inhibitory concentration (MIC) and Fractional inhibitory concentration (FIC) of essential oils alone and in combination:-

The combination effect of oils on *E. coli* and *Micrococcus yunnanensis* (taken as models for further studies) was studied by Checkerboard method.

In **tables (i) and (ii)** the results of combination of rosemary oil and eucalyptus oil are summarized for *E. coli* and *Micrococcus yunnanensis* respectively. In case of *E. coli* when 6.25 µL of rosemary oil was mixed with 50 µL of eucalyptus oil, **0.125±0.01** MIC was obtained. Whereas for *Micrococcus yunnanensis* **0.098±0.01** MIC was obtained when 12.5 µL rosemary oil was mixed with 50 µL eucalyptus oil.

Table i:- Determination of MIC of rosemary oil with eucalyptus oil against *E. coli.*

Concentration of oil	Rosemary oil conc. (µL)					
	100	**50**	**25**	**12.5**	**6.25**	**3.125**
Eucalyptus(50µL)	0.431±0.01	0.279±0.04	0.248±0.03	0.245±0.01	0.125±0.01	0.458±0.02
Rosemary oil alone	1.417±0.2	1.304±0.4	1.186±0.3	1.224±0.4	1.262±0.4	1.313±0.6
Control	0.100±0.01	0.098±0.09	0.100±0.02	0.099±0.05	0.100±0.01	0.100±0.08

Table ii:- Determination of MIC of rosemary with eucalyptus oil for *Micrococcus yunnanensis:*

Concentration of oil	Rosemary oil conc. (µL)					
	100µl	**50µl**	**25µl**	**12.5µl**	**6.25µl**	**3.125µl**
Eucalyptus(50µL)	0.376±0.07	0.294±0.06	0.127±0.03	0.098±0.01	0.122±0.03	0.113±0.01
Rosemary oil alone	1.482±0.2	1.379±0.6	1.263±0.4	1.188±0.4	1.253±0.1	1.246±0.5
Control	0.093±0.1	0.095±0.03	0.092±0.01	0.093±0.02	0.094±0.01	0.094±0.03

In **tables (iii) and (iv)** the results of combination of eucalyptus oil with rosemary oil are shown. For *E. coli* the best MIC value of **0.166±0.02** was depicted by eucalyptus oil (6.25 µL) with rosemary oil (50 µL). **0.144±0.01**values were obtained in case of *Micrococcus yunnanensis* when mixed in ratio of 1:1 with rosemary oil. Eucalyptus oil combination with rosemary oil exhibited better result as compared to eucalyptus oil alone.

Table iii:- Determination of MIC of Eucalyptus oil with rosemary oil for *E. coli.*

Concentration of oil	Eucalyptus oil conc. (µL)					
	100µl	**50µl**	**25µl**	**12.5µl**	**6.25µl**	**3.125µl**
Rosemary(50µL)	0.516±0.01	0.336±0.01	0.254±0.01	0.202±0.01	0.166±0.02	0.178±0.01
Eucalyptus oil alone	1.488±0.3	1.377±0.3	1.238±0.6	1.013±0.2	1.805±0.2	1.728±0.5
Control	0.101±0.03	0.095±0.02	0.101±0.02	0.099±0.01	0.100±0.03	0.101±0.04

Table iv:- Determination of MIC of Eucalyptus oil with rosemary oil for *Micrococcus.*

Concentration of oil	Eucalyptus oil conc. (µL)					
	100µl	**50µl**	**25µl**	**12.5µl**	**6.25µl**	**3.125µl**
Rosemary(50µL)	0.255±0.01	0.144±0.01	0.156±0.8	1.231±0.1	1.195±0.5	1.190±0.5
Eucalyptus oil alone	1.116±0.6	1.006±0.6	1.103±0.3	1.17±0.5	1.156±0.2	1.178±0.5
Control	0.093±0.02	0.095±0.03	0.092±0.01	0.093±0.05	0.093±0.02	0.093±0.01

Determination of Fractional Inhibitory Concentration Indic (FICI):- The FIC value of the combinations was calculated, the results are shown in **table (v).** The combination of rosemary and eucalyptus showed synergistic effect for both the organism.

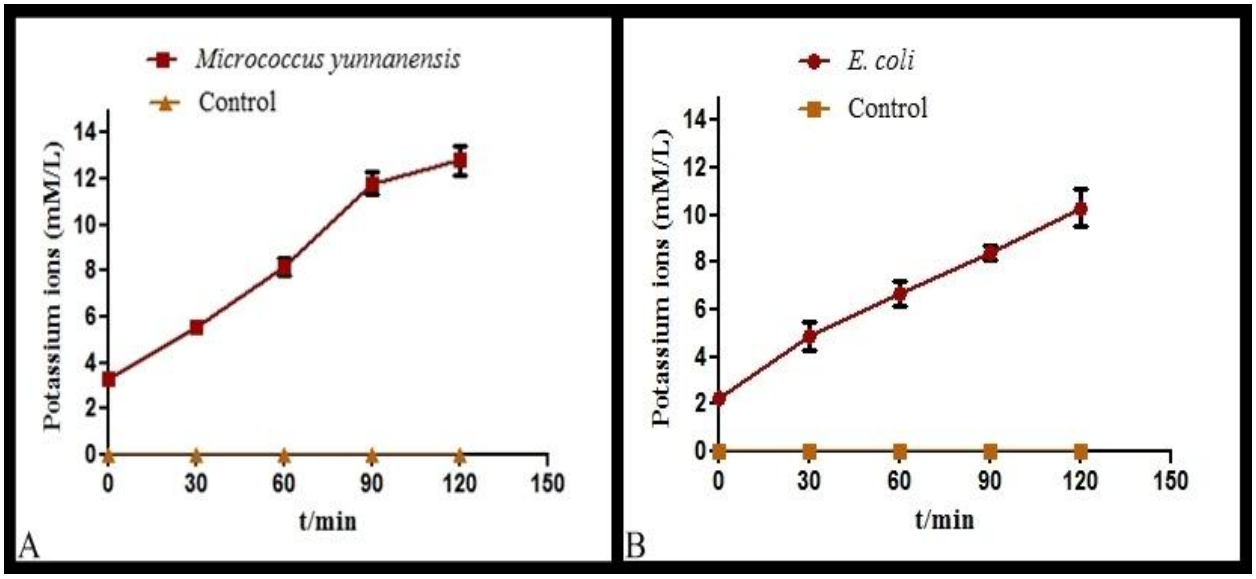
Table v:- Combination effects of rosemary and eucalyptus oil combination against food-borne bacteria using checkerboard titration method

Food borne bacteria	Oil combination		
	Rosemary +Eucalyptus		
	FIC	FICI	Remarks
E. coli	0.105 (Rosemary)	0.268	**S**
	0.163 (Eucalyptus)		
Micrococcus yunnanensis	0.082 (Rosemary)	0.225	**S**
	0.143 (Eucalyptus)		

S: Synergistic; ADD: Additive; ANTA: Antagonistic.

Mechanism of action:- The effect of rosemary and eucalyptus oil combination at their MIC concentration on cell membrane of *E. coli* and *Micrococcus yunnanensis* was studied using two assays:

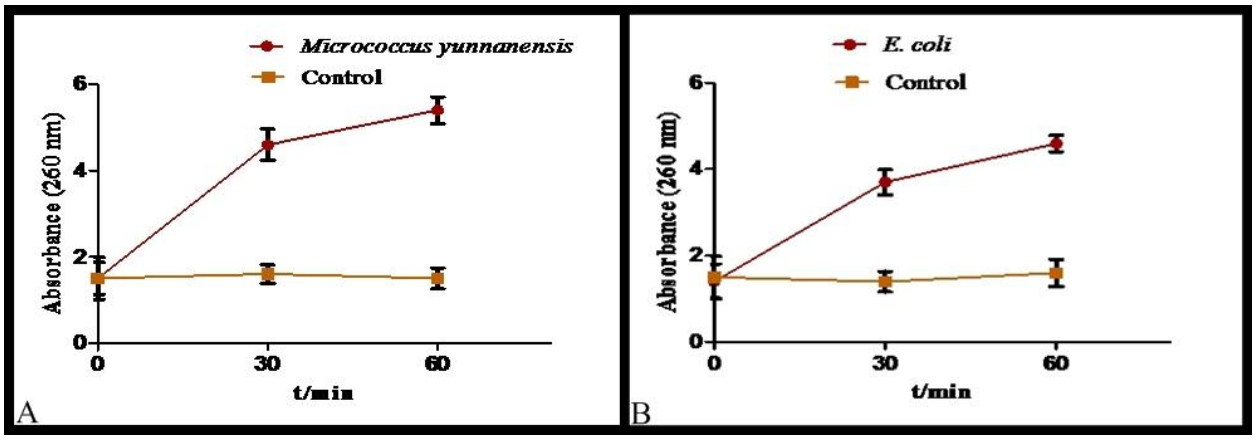
Assay of Potassium ions efflux:- The mode of antibacterial action of combination of oils (rosemary and eucalyptus) at MIC against two tested food borne pathogen was further confirmed using the assay for the release of K^+ ions from treated cells of *E. coli* and *Micrococcus yunnanensis* **(figure i (A, B)**. Treatment of bacterial cells with oil combination at the MIC concentration induced a major efflux of intracellular K^+ ions. The leakage of potassium ions in case of both the bacteria increased with increase in time interval (**figure i (A, B**). However no K^+ ions efflux was observed in control cells of the tested bacteria during the study.



Figures i (A, B):- Effect of Rosemary and Eucalyptus oil combination at MIC concentration on the leakage of potassium ions from the tested food borne bacteria: **A**) *Micrococcus yunnanensis,* and **B)** *E. coli.*

Leakage of Cellular Metabolites:-

The results of the release of cellular material at the absorbance value of 260nm from E. coli and Micrococcus yunnanensis cells exposed to concentration (rosemary + eucalyptus) of oils at the MIC concentrations indicated that the higher exposure time led to higher cell leakage of nucleic acids (**figure ii(A, B**). However, no changes in the absorbance of untreated cells (control) of tested bacteria were seen. At 30 and 60 minutes of treatment, significant increase in the absorbance of the bacterial cells treated with oil combination was demonstrated. The leakage of the material absorbing at 260nm from the bacterial cells treated with combination of rosemary and eucalyptus at MIC, confirms the damage of cell membrane.



Figures ii (A, B):- Effect of Rosemary and Eucalyptus oil combination at MIC concentration on the release rate of material that absorbs at 260 nm from: **A)** *Micrococcus yunnanensis*, and **B)** *E. coli*. Data are expressed as mean values±standard deviations (S.D.), *N*=3. CT=control without treatment.

Discussion:-

Essential oils are natural antimicrobials produced by many aromatic plants and exhibit a great potential to be used in food industry, but there use is restricted due to strong smell and alteration of taste food. To overcome such problem combination of oils can be good option.

In this study commercially available rosemary and eucalyptus essential oils were briefly studied, obtained from local market of Solan, Himachal Pradesh for their potential to be used as food preservative. Rosemary and eucalyptus oil showed potent synergistic interaction (FICI=0.268 for *E. coli* and FICI=0.225 for *Micrococcus yunnanensis*). Similar study on synergism of essential oils was done by **Gibriel *et al.* (2013)** he reported that the combination of rosemary and cumin showed synergistic effect and rosemary and thyme oils combination demonstrated additive effect against *E coli*. In another antibacterial combination study by **Anwesa Bag** and **Rabi Ranjan Chattopadhyay (2015)** on three combinations (coriander/cumin, coriander/ mustard and cumin/mustard) against *Bacillus cereus, Listeria monocytogenes, Micrococcus luteus, Staphylococcus aureus, E. coli and Salmonella typhimurium*, only coriander/cumin combination showed synergistic interaction (FICI: 0.25–0.50) against the studied bacteria except *S. typhimurium* and *M. luteus* where it showed additive effect (FICI: 0.75–0.81). Other tested combinations showed additive effect (FICI: 0.75–2.25) against all the studied bacteria. The results of present investigations depicted that oils when used in combinations gave MIC value at lower concentrations as compared to MIC value of oil when used alone .These findings collaborates with illustrations of **Tajkarimi *et al.* (2010)** who stated use of less concentration of essential oils combination in food stuff to avoid the sensory impact of higher concentration and also providing the food safety is the foundation of usage of essential oil combination.

One of the indicator of increased permeability and loss of viability by cells is the loss of potassium ions into extracellular space, confirmed by various studies of **Cox *et al*. (2001)** and **Bouhdid *et al.* (2010).** In this study, we report the effect of rosemary and eucalyptus oil combination on *Micrococcus yunnanensis* and *E. coli*. K+ release during exposure to the oil combination was measured in both strains, and was found to be induced at MIC concentration. The release of potassium ions was observed to increase with time interval in case of both bacteria. Therefore, it can be concluded from results that rosemary and eucalyptus oil combination interact with cellular membranes of bacteria, changing their permeability to cations like K+. These results collaborates with illustrations of various researchers namely, **Bajpai *et al.* (2015)** examined the effect of *Ginkgo biloba* essential oil on *B. cereus* and *E. coli*, and evaluated that oil caused release of potassium ions; **Bouhdid *et al*. (2010)** noticed the increase in potassium ion concentration extracellularly in case of *S. aureus* and *P. aeruginosa* when exposed to *Cinnamomum verum* oil; **Trombetta *et al*. (2005)** who reported that oregano essential oil cause potassium ion release in *S. aureus* and *P. aeruginosa*.

E. coli and *Micrococcus* suspension treated with combination of rosemary and eucalyptus oil at MIC, lost significant 260 absorbing materials with increase of time interval, suggesting that nucleic acids and certain protein were lost through a damaged cytoplasmic membrane. Similar studies was carried out by **De Souza et al. (2013)** who studied the effect of rosemary and oregano oil on *Pseudomonas aeruginosa*, when used alone and in combination. It was

observed that immediately after the contact of essential oil alone or in combination with bacteria caused leakage of 260 nm absorbing material. **Ifesan et al. (2009)** observed the leakage of 260nm absorbing material by *S. aureus* when exposed to Eleutherine Americana. **Carson and Riley *(2002)*** showed leakage of 260nm absorbing material by *S. aureus* when treated with tea tree oil.

Conclusion:-

The present study confirms the antimicrobial action of rosemary and eucalyptus oil combination on the permeability of cell membrane at its MIC conc. on the tested bacteria. It leads to the loss of some cellular components from the cell to outside. In future studies should be dedicated to see the implication of essential oil combination treatment to different food matrices. The toxicology study of the combination should also be carried out.

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