Journal of Animal Research: v.8 n.6, p. 989-998. December 2018

DOI: 10.30954/2277-940X.12.2018.7

In-vitro Assessment of Antimicrobial, Antibiofilm and Antioxidant Potential of Essential Oil from Rosemary (*Rosmarinus officinalis* L.)

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Received: 08 Oct., 2018 **Revised:** 25 Oct., 2018 **Accepted:** 30 Oct., 2018

ABSTRACT

The present study was conducted to investigate *in-vitro* antimicrobial, antibiofilm and antioxidant efficacy of Rosemary essential oil (REO) for its potential application in meat products. The oil was tested against four Gram positive (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus and Enterococcus faecalis*) and six Gram negative (*Salmonella enterica serovar Typhi, Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, Proteus mirabilis* and *Klebsiella pneumoniae*) organisms using zone of inhibition and Minimum Inhibitory Concentration (MIC) estimation. Widest inhibition zone was exhibited by *Staphylococcus aureus* whereas, *Enterococcus faecalis* and *Salmonella enterica* showed the largest MIC values. Antibiofilm activity (%) was determined by using pure culture of *L. monocytogenes* and *S. aureus* as positive control, whereas 1, 1 diphenyl-2picrylhydrazyl (DPPH) and 2-2-azinobis-3ethylbenthiazoline-6-sulphonic acid (ABTS) radical scavenging activity was taken as parameter for antioxidant activity. The results showed that with application of REO, biofilm formation of both *L. monocytogenes* and *S. aureus* was inhibited by 73.0 and 77.65 %, respectively in comparison to their respective controls. Six different concentrations of oil was used for determination of ABTS and DPPH radical scavenging activity and it was ranging from 8.16- 51.80% for DPPH whereas ABTS values ranged from 6.81-44.16% for rosemary oil under investigation. It can be concluded that rosemary essential oil possesses potent antimicrobial, antibiofilm and antioxidant activity, and may be used as a natural preservative to extend the storage stability of meat products.

Keywords: Rosemary essential oil, antimicrobial activity, DPPH, ABTS, antibiofilm activity

A key source for high quality nutrients, meat and meat products occupy a prominent position in human food basket (Mehta *et al*., 2015). One of the prominent limitations is perishability and susceptibility of meat to bacterial spoilage and oxidative degradation (Fung and Toldra, 2010). To counter that, application of synthetic antimicrobials and preservatives in meat products is usually done. However, with increase in consumer awareness about their health and well-being, negative perception towards synthetic antimicrobials and preservatives has led to an extensive search for natural sources. Along with containment of growth of spoilage microbes, the prime consideration is to cease oxidation reactions that often occur during meat processing and storage which further leads to deterioration of quality as detected by off-flavours, decreased nutrient value and health concerns due to accumulation of toxic compounds (Lorenzo *et al*., 2014). Further, some food related pathogens are known to form stable biofilms in meat processing plants which may be a potential source for contamination that leads to meat spoilage and also allows pathogens to tolerate and withstand hostile and adverse environmental conditions. It is reported to render protection to bacteria against various antimicrobials and sanitizers, thereby increasing resistance in them by at least 1000 times than normal (Miladi *et al.,* 2016).

Utilization of natural antimicrobials and antioxidants can be foreseen as one of the most important strategies for development of functional and healthier meat products. In this context, essential oils and extracts of medicinal herbs and spices have attained value due to the inherent antimicrobial as well as antioxidant properties (Fernandes *et al*., 2018; Poojary *et al*., 2017; Putnik *et al*., 2017). Many studies have explored the application of aromatic phyto-chemical preparations with two fold functionality against microbial degradation as well as lipid oxidation in meat and meat products, particularly essential oils (Govaris *et al*., 2005). Essential oils (EOs) are naturally-derived aromatic compounds, with broad range of biological activities (El-Asbahani *et al*., 2015). Up to now, EOs have been used as flavouring additives in food products but recently they are proving to be potent source for natural antimicrobials in food and beverage products (Burt, 2004).

Rosemary (*Rosmarinus officinalis* L.) belongs to *Lamiaceae* family and chemically it can be classified as one of three chemotypes i.e. cineoliferum (high 1, 8-cineol content), camphoriferum (camphor >20%) and verbenoniferum (verbenone >15%) (Napoli *et al*., 2010). It has been reported to have many beneficial effects such as antiviral, anti-inflammatory and anti-carcinogenic. The aim of this study was to investigate and evaluate *in-vitro* antimicrobial, antibiofilm and antioxidant potential of rosemary essential oil for its possible application in meat products replacing synthetic antimicrobials and antioxidants.

MATERIALS AND METHODS

Source of rosemary essential oil

Rosemary essential oil was procured from Kanta Enterprises Pvt. Ltd, Noida, UP, India. As per the certificate of analysis provided with the product, it has specific gravity in range of 0.894 to 0.912 and refractive index in range from 1.46 to 1.48. All the reagents and chemicals used in the study were of analytical grade and procured from reputed firms.

Bacterial strains and growth conditions

Ten pure freeze dried cultures were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India viz. *Bacillus cereus* (MTCC 1272), *Enterococcus faecalis* (MTCC 890), *Escherichia coli* (MTCC 723), *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 96), ,*Salmonella enterica serovar Typhi* (MTCC 733), *Shigella flexneri* (MTCC 1457), *Pseudomonas aeruginosa* (MTCC 74), *Proteus mirabilis* (MTCC 425) and *Klebsiella pneumoniae* (MTCC 109). These cultures were revived and stock cultures were prepared and maintained at -80° C by regular passaging.

Estimation of antimicrobial activity

Antimicrobial potential of Rosemary essential oil (REO) was estimated against Gram-positive (*Bacillus cereus* MTCC 1272, *Enterococcus faecalis* MTCC 890, *Listeria monocytogenes* MTCC 1143 and *Staphylococcus aureus* MTCC 96) and Gram-negative (*Salmonella enterica serovar Typhi* MTCC 733, *Escherichia coli* MTCC 723, *Shigella flexneri* MTCC 1457, *Pseudomonas aeruginosa* MTCC 741, *Proteus mirabilis* MTCC 425 and *Klebsiella pneumoniae* MTCC 109) pathogens through modified agar-well diffusion assay (Presti *et al.,* 2015; Aneja *et al.,* 2011). For anti-bacterial assay, streaking of fresh overnight culture of each pathogen over BHI agar plate was done and the plates were incubated at 37^{o}C for 16h. Under aseptic conditions, pure isolated colonies was transferred to sterile normal saline (0.85%) solution and the density of each microbial suspension was adjusted equal to 0.5 McFarland standard (Andrews, 2001). Thereafter, 100µl of the inoculum of each test pathogen was spread over pre-solidified MHA (Muller Hinton Agar) plates and 8mm wells were made using a sterile borer after drying. 100 µl of Rosemary essential oil (REO) was poured into each of the wells and in order to accelerate its diffusion in agar, the plates were pre-incubated at 4^{o}C for 1hour, followed by overnight incubation at 37^{o}C. The antibacterial activity, indicated by the zone of inhibition (ZOI) surrounding the well containing REO was recorded using zone scale (Hi-Media). All the tests were performed in triplicate, and the mean values of the diameter of inhibition zones were recorded.

The Minimum Inhibitory Concentration (MIC) values were determined by method followed by Jiang *et al*. (2011) with slight modifications. Final concentration of REO (0.5% v/v) was made by dissolving it in sterilized physiological saline solution (0.9% w/v) added with

Tween 80. Serial double dilutions of the REO were prepared in a 96-well microtiter plate in the range 0.156% to 4.0% (v/v). The final concentration of each bacterial strain in nutrient broth was adjusted to 10^5–10^6 CFU/mL. Each REO dilution (100 µL) was dispensed into the wells of a microtiter plate and each well was then inoculated with 200 µL of the suspension which contained equal amounts of nutrient broth and bacterial culture. The resulting suspensions were mixed with a micro-pipette and microtiter plates were incubated at 37 °C for 24 h. After incubation, the wells were examined for growth of microorganisms and the MICs were determined. The MIC was computed as the lowest concentration of the essential oil at which the microorganism did not demonstrate visible growth.

Antibiofilm activity of Rosemary essential oil: Inhibition of biofilm growth and development and its visualization

Biofilm inhibition was achieved by following method of Galvao *et al*. (2012). 10 µL of a standardized (~10^6 CFU/ml) *L. monocytogenes* and *S. aureus* culture was aliquoted into each 96-well microtitre plate followed by the addition of 30 µL of REO (1250 ppm), respectively. Following incubation at 37°C for 24 h, the plates were rinsed three times with phosphate-buffered saline (PBS, pH 7.2) to remove loosely attached cells. The plates were air-dried and then the wells were stained with 250 µL of 0.1% crystal violet and incubated at room temperature for 30 min. After incubation, the plates were washed and then left to dry. Finally, 250 µL of 33% glacial acetic acid was added to solubilize the dye and the OD_{570} was recorded using microplate reader. The per cent biofilm inhibition was estimated following the formula: anti-biofilm activity (%) = (Control $_{OD570\ nm}$ − Test $_{OD570\ nm}$ / Control $_{OD570\ nm}$) × 100. The positive control was the amount of biofilm formed with a pure culture of *L. monocytogenes* and *S. aureus*.

Visualization of biofilms

L. monocytogenes and *S. aureus* biofilms, with or without treatment with REO, were grown on pre-sterilized glass cover slips (Musthafa *et al*., 2010). An aliquot of 80 µL/well (~10^6 CFU/mL) of overnight grown test pathogens were added to 6 well tissue culture plate containing sterile glass cover slips, BHI medium (1680 µL/well) and REO (240 µL/well). The bacterial suspensions were used as controls. Biofilms were stained with crystal violet and the stained biofilms were visualized under light microscope at 40X magnification (Olympus Microscope, USA).

Antioxidant activity of Rosemary essential oil (REO)

Antioxidant activity of REO was evaluated as free radical scavenging activity (RSA). Ability of essential oil to donate hydrogen atoms or electrons was measured from bleaching of coloured methanoloic solution of 1, 1 diphenyl-2picrylhydrazyl (DPPH). More the number of hydroxyl groups, the higher is the possibility of free radical scavenging ability.

1, 1 diphenyl-2picrylhydrazyl radical scavenging activity (DPPH)

Radical scavenging potential of Rosemary essential oil (REO) was assessed using a methanolic solution of the stable free radical i.e. DPPH. The method of Blois (1958) was used in studying the effect of various oil concentrations on DPPH radicals with some modifications. A solution of DPPH (0.15 mmol/L) in methanol was prepared. Oil concentrations (0.125%, 0.25%, 0.5%, 1.0%, 1.25%, 1.5%, 2.0%, and 2.5%) were prepared in methanol and 200 µl of each dilution was mixed with 50 µl of DPPH solution in a 96-well microtitre plate. The mixture was allowed to stand at room temperature in dark for 30 min and the decrease in absorbance at 517 nm was measured. Butylated hydroxyanisole (BHA) was used as positive control. Inhibition of DPPH radical was calculated by the following formula:

$$\% \text{ Radical Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 and A_1 are the absorbance of control and the sample, respectively. The IC_{50} value, which represents the concentrations of the sample required to cause 50% inhibition of DPPH radical, was estimated by linear regression analysis from the obtained RSA values and was expressed in µl of essential oil per ml.

2-2-azinobis-3ethylbenthiazoline-6-sulphonic acid radical scavenging activity (ABTS)

It was determined by method followed by Yang *et al*., (2010) and Kumar *et al*., (2017) with slight modifications**.** ABTS cation decolorization assay was conducted on various concentrations of Rosemary essential oil (REO) viz. 0.125%, 0.25%, 0.5%, 1.0%, 1.25%, 1.5%, 2.0% and 2.5%, made in methanol. 50 µl of various concentrations of REO and 150 µl of ABTS solution were mixed and after 1 min incubation at room temperature, absorbance was measured spectrophotometrically at 732 nm. Methanol was used as blank solution, and the ABTS solution without essential oil served as the control. The cation scavenging activity was measured same as with DPPH and A_0 and A_1 are the absorbance of control and the sample, respectively.

$$\% \text{ Radical Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Statistical analysis

Data was analyzed statistically on 'SPSS-16.0' (SPSS Inc., Chicago, II USA) software package as per standard methods (Snedecor and Cochran, 1989). Whole set of experiment was repeated three times and the mean values were reported along with standard error. The statistical significance was estimated at 5% level ($p<0.05$) and evaluated with Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Antimicrobial activity and MIC of Rosemary essential oil

The results obtained from the well- diffusion assay indicated that REO exerted antibacterial activity against all the tested strains (Fig. 1). The size of the inhibition zone of REO varied from 12.5 to 36.0 mm and maximum inhibition diameter was observed for *Staphylococcus aureus* (36±0.68) followed by *Listeria monocytogenes* (26.5±0.50), whereas minimum zone diameters were observed in *Salmonella enterica* (12.50±0.26) followed by *Enterococcus faecalis* (12.75±0.41) (Fig. 2). The results of zone inhibition assays corresponded to the MIC values determined by microdilution broth assay. The MIC (ppm) values of rosemary essential oil against all the targeted organisms were found in range of 1250 to 25000 ppm (Fig. 3). A similar trend between MIC values and zone inhibition reveals that *Staphylococcus aureus* responded maximum to REO. *Staphylococcus aureus* is a common gram positive organism responsible for food borne illness around the globe. Further, it is a highly changeable pathogen in response to antibiotics and can put human health in jeopardy (Alfatemi *et al*., 2014). Santoyo *et al*. (2005) investigated antimicrobial activity of supercritical CO_2 extracted essential oil–rich fractions obtained from *Rosmarinus officinalis* L. They conducted their studies through disc diffusion and broth dilution methods against six microbial species viz. *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. All components of the essential oil presented antimicrobial activity against tested microorganisms, with inhibition zones and minimal bactericidal concentration values varied from 17 to 33 mm and 2.25 to 0.25 mg/ml, respectively. Similar to our findings, they reported that *S. aureus was* the most sensitive microorganism, with maximal inhibition zones (27 to 33 mm) and the lowest MBCs (0.75 to 0.25 mg/ml). Fu *et al*. (2007) studied the antimicrobial activity of the clove (*Syzygium aromaticum* L.) and rosemary (*Rosmarinus officinalis* L.) essential oils alone and in combination and found that the MICs of clove oil and rosemary oil ranged from 0.062% to 0.500% (v/v) and 0.125% to 1.000% (v/v), respectively. Further, a synergism in antimicrobial action between these two oils was observed against individual tested microorganisms.

The results obtained in this study for MICs of rosemary essential oils are in accordance with findings of Fu *et al*. (2007) and Ojeda-Sana *et al*. (2013) who reported the MIC range from 1.0 to 2.5% against tested food borne pathogens. Mohsenabadi *et al*. (2018) evaluated the antimicrobial properties of starch-carboxy methyl cellulose film incorporated with chitosan nanogel encapsulated rosemary essential oil and found that REO resulted in significant inhibition against *Staphylococcus aureus*. Honorio *et al*. (2015) studied *in-vitro* antimicrobial efficacy of two essential oils viz. *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO) along with its major compounds such as carvacrol (CAR) and 1,8-cineole (CIN), respectively. They reported that minimum inhibitory Concentration (MIC) of both OVEO and CAR was 1.25 µl/ml, while for ROEO and CIN the

Fig. 1: Zone of inhibition assay (mm) of Rosemary Essential Oil (REO) against food spoilage microorganisms

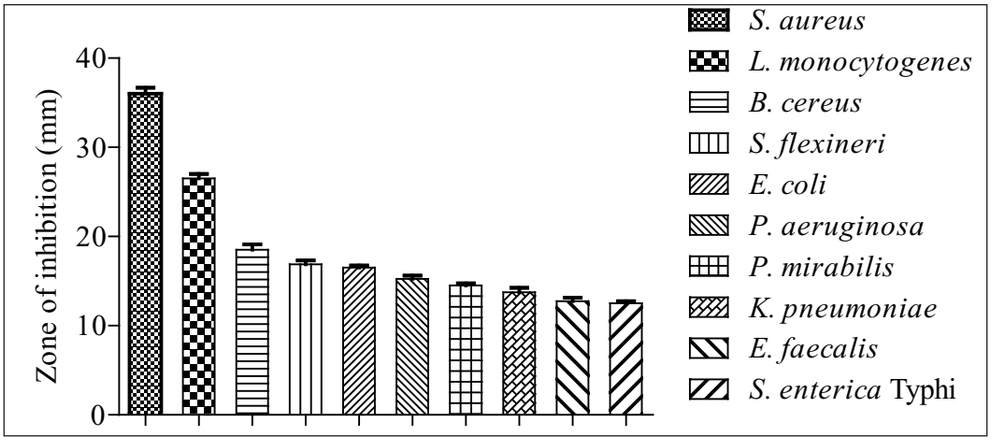

Fig 2: Zone of inhibition assay (mm) of Rosemary Essential Oil against ten food spoilage microorganisms (Mean ± S.E.), n=3

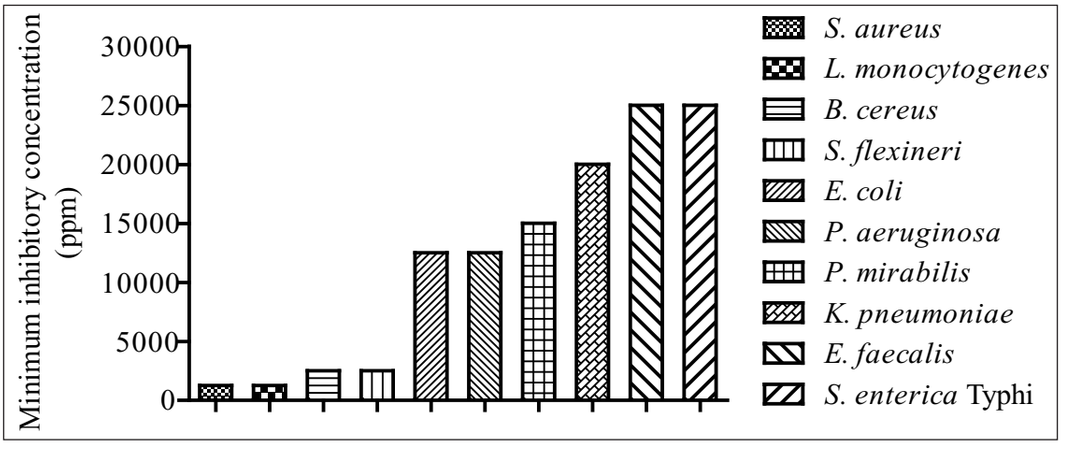

Fig. 3: Minimal Inhibitory Concentration (ppm) of Rosemary Essential Oil against ten food spoilage microorganisms (Mean values), n=3

MIC value was 10 µl/ml against tested organism i.e. *S. aureus*. Similarly, Jiang *et al*. (2011) conducted the antimicrobial activity estimation of REO and its main component; α-pinene and found MICs in range from 0.03% (v/v) to 1.0% (v/v) and 0.3% (v/v) to 4.0% (v/v), respectively against tested microorganisms.

In the present study, a strong positive correlation between MIC values and zone inhibition diameters was observed. However, little discrepancy of obtained values as compared with previously conducted studies might be due to variation in origin and type of rosemary plant, method of extraction employed, final composition of rosemary essential oil, methodology adopted for evaluation of zone of inhibition and MIC etc. Zaouali *et al*. (2010) also demonstrated a significant variation in the composition of oils and their biological activity potentials according to varieties and populations of plants. Perusal to results presented in Fig. 2 and 3, tested REO was found to be more effective against gram-positive bacteria than gram-negative. These differences could be attributed due to fact that the cell membrane of gram positive bacteria contains lipoteichoic acids which might have facilitated the penetration of hydrophobic compounds of essential oil, whereas presence of an extrinsic membrane made up of lipopolysaccharides surrounding the cell wall of gram negative bacteria, might have limited the diffusion rate

of hydrophobic compounds. It would have made them inherently resistant to these hydrophobic and lipophilic compounds (Rodriguez-Garcia *et al*., 2016; Tongnuanchan and Benjakul, 2014; Burt, 2004).

Antibiofilm activity of Rosemary essential oil

Some of the microorganisms like *Listeria* have a tendency to exist as communities enclosed in matrix, predominantly polysaccharides in nature, known as biofilms. It enables them to exhibit resistance against sanitizers, disinfectants and antimicrobial agents. They can be formed on wide range of surfaces in food processing industry and are of a great concern from safety point of view as bacteria from biofilms can be transferred to food products (Gandhi and Chikindas, 2007). In present study, antibiofilm potential of REO was tested against two organisms viz. *L. monocytogenes* and *S. aureus.* On exposure to essential oil, a significant reduction in biofilm formation by bacteria was observed. The results showed that in the presence of REO, biofilm formation of both *L. monocytogenes* and *S. aureus* was inhibited by 73.0% and 77.65%, respectively in comparison to their respective controls (Fig. 4). The correlation between anti-microbial efficacy and anti-biofilm activity was observed. Furthermore, with the addition of the REO, *L. monocytogenes* and *S. aureus* exhibited a lower degree of cluster cells with poorly developed biofilm architecture than control group. The control slides demonstrated a dense layer of biofilm formation and were easily stained with CV stain. The images clearly differentiated the biofilm architecture between treated and the untreated biofilms in both the tested pathogens. Results of microscopic analysis revealed the maximum degree of reduction in the number of micro colonies at the concentration of 1250 ppm against both the tested pathogens (Fig. 4).

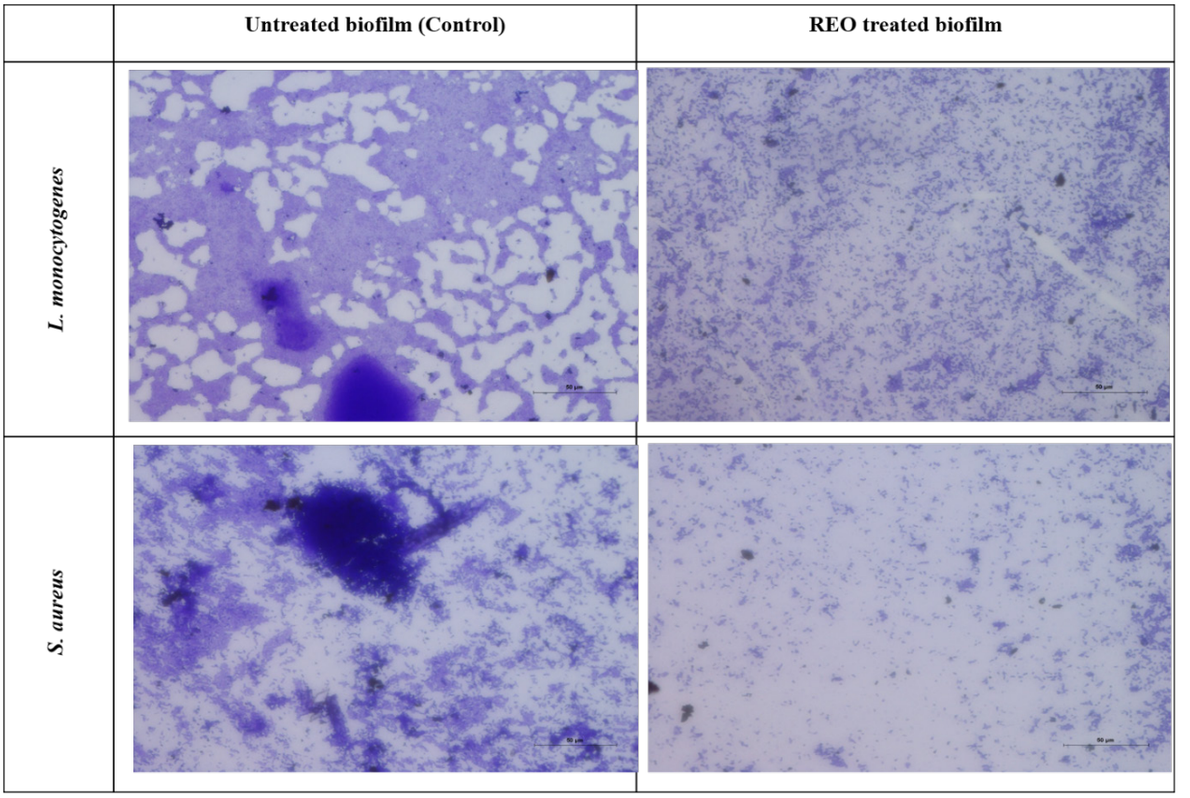

Fig. 4: Light microscopic images of biofilms of *L. monocytogenes* and *S. aureus* grown in the absence and presence of rosemary essential oil (REO)

Antioxidant efficacy of Rosemary essential oil

The results for antioxidant activity (DPPH and ABTS) of Rosemary essential oil are presented in Table 1.

Table 1: DPPH and ABTS Radical Scavenging activity of Rosemary essential oil (Mean±S.E.), n=3

Sl. No.	Tested Concentrations (ppm)	DPPH Radical Scavenging Activity (%)	ABTS Radical Scavenging Activity (%)
1	1250	8.16±0.30	6.81±0.31
2	2500	12.50±0.42	11.16±0.47
3	12500	29.60±0.42	21.83±0.30
4	15000	38.00±0.44	29.50±0.56
5	20000	42.00±0.36	36.16±0.47
6	25000	51.80±0.47	44.16±0.70

A solution of DPPH• (0.15 mmol/L) in methanol was used along with five different concentrations of oil (1250, 2500, 12500, 15000, 20000 and 25000 ppm). The concentrations of essential oil to be tested were selected on the basis of MIC values as depicted in Fig. 3. There was an incremental trend of radical scavenging activity with increasing concentration of oil and it varied from 8.16% to 51.80% for DPPH and 6.81% to 44.16% for ABTS at tested concentrations. The higher radical scavenging activity of oil could be due to presence of active principles i.e. Myrcene, α-pinene etc. IC_{50} value, which is defined as concentration of antioxidant required to scavenge 50% of DPPH present in test solution was recorded to be 66.6 µl/ml. A comparison was made with synthetic antioxidant i.e. BHA and it exhibited an IC_{50} value of 25.0 µg/ml. It signifies that 1 µg of BHA is equivalent to 2.7 µl of tested REO. Ojeda-Sana *et al*. (2013) reported a significant positive correlation between chemical composition of rosemary essential oil and its free radical scavenging capacity. Myrcene rich chemotype of rosemary essential oil exhibited better free radical scavenging capacity and IC_{50} values ranging from 11 µL/mL and 25 µL/mL. Yang *et al*. (2010) evaluated the antioxidant activities of major components of six different herb essential oils and found that ABTS radical scavenging activity was 69.5± 2.82% with RC_{50} value at 3% concentration of rosemary essential oil. The above evidence from previous studies suggest that some essential oils have potential antioxidant and free radical scavenging activity. Along with proven health benefits, these attributes can be helpful in preventing oxidative rancidity in high fat food products such as meat, thereby extending its storage stability.

CONCLUSION

In vitro antimicrobial, antibiofilm and antioxidant study of Rosemary essential oil reveals that it has shown strong antimicrobial action against common food spoilage microorganism with concurrent broad spectrum of activity against Gram positive organisms, capability to inhibit formation of biofilm and it also possess potential ability to scavenge free radicals. Owing to this action, it can be used as an alternative to the synthetic antimicrobials and antioxidants for control of foodborne pathogens, which can minimize the health risk and side effects. Further, it can be tried as a potential natural preservative in foodstuffs, particularly meat and meat products without affecting the organoleptic quality and can prevent the oxidative rancidity during storage at refrigeration temperature, thereby enhancing the storage stability and shelf life. Even so, there is a need to utilize essential oils for incorporation in food matrices for multifunctional benefits, its *in-vivo* toxicological studies in animal model systems can be carried out in future.

ACKNOWLEDGEMENTS

Authors are grateful to Ministry of Food processing Industries (MoFPI), Government of India and Science and Engineering Research Board (SERB) for financial support in form of extramural research grant (SERB/MOFPI/0025/2014).

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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