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EVALUATION OF ANTIBACTERIAL ACTIVITY OF *ROSMARINUS OFFICINALIS L.* ESSENTIAL OIL

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ABSTRACT

The antibacterial activity of crude and essential oil extract of rosemary (*Rosmarinus officinalis* L.) against the infectious bacteria viz., *Escherichia coli, Bacillus subtilis, Pseudomonas* spp and *Staphylococcus aureus* were evaluated *in vitro*. It was found that rosemary oil exhibits high antibacterial activity against *E. coli, B.subtilis, S.aureus,* and *Pseudomonas* spp, when compared to crude extract. The *in vitro* Minimum Inhibitory Concentration (MIC) of rosemary oil was found to be 100µg ml^{-1} for inhibiting the growth of *Pseudomonas* spp; 50µg ml^{-1} for inhibiting the growth of *B. subtilis* and *S. aureus* whereas, it was found to be 12.5µg ml^{-1} for *E. coli.* The statistical analysis for MIC of *R. officinalis* oil was found to be 42.82µg/ml for inhibiting the growth of *B.subtilis* and *S. aureus*, it was found to be 10.88µg/ml for *E.coli* and 85.66µg/ml for *Pseudomonas* spp.

KEYWORDS: Antibacterial activity, *Bacillus subtilis*, Essential oil, *Escherichia coli*, *Rosmarinus officinalis*, *Pseudomonas* spp, *Staphylococcus aureus*

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INTRODUCTION

Rosmarinus officinalis L. plant commonly known as Rosemary belongs to the family *Labiateae.* The plant is widely used in Mediterranean dishes as a spice. It is used in the manufacturing of soaps and other cosmetics as it gives good fragrance. The leaf extracts of rosemary are proposed as an important human dietary factor, and it is also investigated as a therapeutic agent against several diseases (Al-Sereiti et al., 2009). It is commonly used in ethnomedicine as general stimulant, for improvement of circulation, treatment of rheumatic pains, hyperglycemia, skin care, antioxidant, antimicrobial properties and also increases bone density (Hamedo, 2009). The disease causing bacteria that have become resistant to antibiotics are causing an increasing public health problem due to the continuous use of antibiotics. The natural extract from plants can be alternatively used to antibiotics as they do not enhance antibiotic resistance and are screened for their potential uses as alternative remedies for the treatment of many infectious diseases (Tepe *et al*. 2004). Prabu seenivasan *et al* (2006) has reported the use of extracts from medicinal and aromatic plants as a major source of natural organic compounds in drug production. The present investigation was undertaken with the aim of finding out the efficacy of *R.officinalis* crude and essential oil extract against infectious Gram positive (+) and Gram negative (-) bacteria.

MATERIALS AND METHODS

The *Rosmarinus officinalis L.* an aromatic and medicinal plant was chosen for the present study. The pure cultures of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Pseudomonas spp*., were procured from the stock cultures of Molecular Biology department, Bangalore University, Bangalore.

Preparation of Plant aqueous extract and Essential oil

An aqueous crude extract was prepared following the method of Ateyyat *et al*. (2009) by boiling 10% (w/v) of the air dried leaf powder in sterile distilled water for 10 min and cooled to room temperature over night. The aqueous extract was filtered using a Millipore filter to remove particulate matter and the final volume was adjusted to 100 ml with distilled water. The rosemary oil was extracted from *R.officinalis* using Baratta (1998) method. The different concentration of the oil was obtained by diluting it with Triton X 100.

Antibacterial Activity

Screening of crude and essential oil *R. officinalis* for antibacterial activity was performed using the Agar well diffusion assay. The bacterial inoculums was swabbed on to the Muller Hinton Agar plates and 100µl of undiluted, 1:1 and 1:2 dilutions of crude and essential oil were added on to 8mm diameter well bored on these plates and 5mg/ml streptomycin was added as a positive control. The plates were incubated at 37^0C for 18hrs and then the zone of inhibition was measured. The Minimum Inhibitory Concentration (MIC) was performed for essential oil following the procedure of Deutsches Institut fur Normung (1998) and the concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.124 µg ml^{-1} was done using the 2- fold serial dilution technique. The bacterial turbidity was matched with 0.5 Mc Farland's standard (10^6–10^7 cfu mL^{-1}) and 100µl of this culture were added to 10 mL of the above mentioned dilutions. The tubes were mixed well and incubated at 37^0C, 120 rpm for 18 h and turbidity was checked to determine MIC. Streptomycin was used as a positive control for both the Agar well diffusion assay and MIC. The 3-way ANOVA statistical analysis for *in vitro* studies of Agar well diffusion assay was done using IBM–SPSS (Statistical Package for Social Science) version 20. The *in vitro* value obtained for MIC was analyzed and the exact MIC value was statistically calculated by

applying the Logistic Regression Model, which is given below.

$$\pi(X) = P(Y=1) = e^{\beta 0+\beta 1X} / 1+ e^{\beta 0+\beta 1X}$$

Where, Y=Response (Binary outcome-Turbidity/ No Turbidity); X=Concentration (Covariate); β_0 and β_1=Regression Coefficients; βo = -68.745, β_1=1.836 for *B. subtilis* and *S. aureus;* βo = -61.857, β_1=6.598 for *E.coli* and βo = -68.218, β_1=0.912 for *Pseudomonas* spp.

Here, first the Logistic Regression Model was fitted and later we used this model to find the value of 'x' (concentration), for which, P[Y=1] ≈ 1, i.e., the probability where there will be no turbidity is approximately equal to one. The above analysis was carried out using R software.

RESULTS AND DISCUSSION

The antibacterial activity of *R.officinalis* crude extract and essential oil against two Gram-positive and two Gram-negative bacteria performed using Agar well diffusion method is depicted in table 1, Fig 1, 2, 3 and 4. The analysis of the results revealed that the concentrated crude extract had less inhibitory effect on all the bacteria tested such as *E.coli, Pseudomonas spp, S.aureus* and *B. subtilis* (Fig 7). It was found that the 1:1 and 1:2 dilutions of the crude extract had no effect on all the four bacteria tested (Fig 5). This may be due to low concentration of active compounds in the crude extract. The undiluted essential oil exhibited antibacterial activity against all the four bacteria tried. The maximum inhibition zone was found in *E.coli* (25mm) followed by *B.subtilis* (24mm), *S. aureus* (20mm) and *Pseudomonas* spp (17mm). The inhibition zones of all the bacteria decreased with the dilutions (Fig 6 and 8). The present findings agrees with Sue *et al* (2000) who have reported inhibitory effects of 45 essential oils on eight Gram positive and Gram negative bacteria but, does not agree with the findings of Shigeharu *et al* (2001) who had reported weak antibacterial activity of essential oils against Gram-negative bacteria. The 3-way ANOVA statistical analysis of the Agar well diffusion of crude and essential oil extract shows 'P' value which is less than 0.05 for all the parameters such as bacteria versus extract, bacteria versus concentration, extract versus concentration and bacteria versus extract versus concentration. These values indicate that all the results obtained are unique and significant. (Table 2)

The analysis of the results of Minimum Inhibitory Concentration (MIC) of different dilutions of essential oil is presented in Table 3 and Fig 9. The *in vitro* MIC value indicates that low concentration (12.5µg/ml) of essential oil was very effective against most infectious Gram negative bacteria, *E.coli.* It was found that 50µg/ml exhibited inhibitory activity against Gram positive bacteria, *S.aureus* and *B.subtilis*. The *Pseudomonas* spp exhibited MIC of 100µg/ml, indicating a weak inhibitory activity. The actual MIC values were calculated statistically by applying a Logistic Regression Model and found to be 10.88µg/ml for *E.coli*, 85.66µg/ml for *Pseudomonas* spp and 42.82µg/ml for *S.aureus* and *B.subtilis*. The present findings are in accordance with the findings of Moghtader & Afzal (2009) and also with Lodhia *et al*; 2009 who have reported the antimicrobial activity of Rosemary against both Gram-positive and Gram-negative bacteria. The antimicrobial activity of Rosemary may be due to the presence of chemicals that constitute its essential oil. The major chemical component found in rosemary oil is α-pinene followed by 1, 8 – cineole, camphene, β-myrcene, camphor and borneole (Jamshidi et al., 2009, Moghtader & Afzal, 2009). The antimicrobial activity of essential oils is exhibited due to its active components which partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering more permeability as reported by Knobloch *et al* (1986) and Sikkema *et al* (1994). In the present investigation, the extensive leakage from bacterial cells or the exit of critical molecules and ions may have led to bacterial death

as described by Denyer and Hugo (1991). The present investigation together with the previous studies provides support for the modulation of new therapeutic drugs. Further, additional *in vivo* studies and clinical trials would be needed to justify and evaluate the potential of rosemary oil as an antibacterial agent in topical or oral applications.

Table 1
Showing the antibacterial activity of the crude extract and essential oil of Rosmarinus officinalis L., by Agar well diffusion method.

Bacteria	Diameter of zone of inhibition in millimeter							
	Plant crude extract				Essential oil extract			
	S	A	B	C	S	A	B	C
E.coli	22	13	-----	-----	22	25	20	15
Bacillus subtilis	24	7	-----	-----	24	21	18	15
Pseudomonas spp	25	12	-----	-----	25	17	10	-----
Staphylococcus aureus	25	11	-----	-----	25	20	15	10

S – +ve control (Streptomycin); A – undiluted sample; B – 1:1 dilution; C – 1:2 dilutions

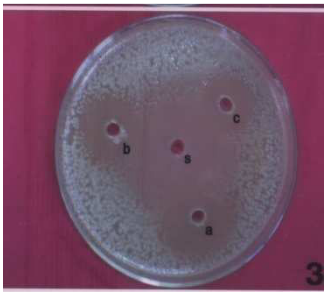

Figure 1
Agar well diffusion of Rosemary oil against *E.coli*

Figure 2
Agar well diffusion of Rosemary oil against *B.subtilis*

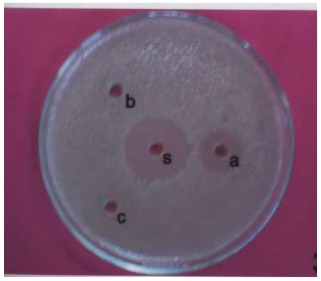

Figure 3
Agar well diffusion of Rosemary oil against *Pseudomonas* spp

Figure 4
Agar well diffusion of Rosemary oil against *S.aureus*

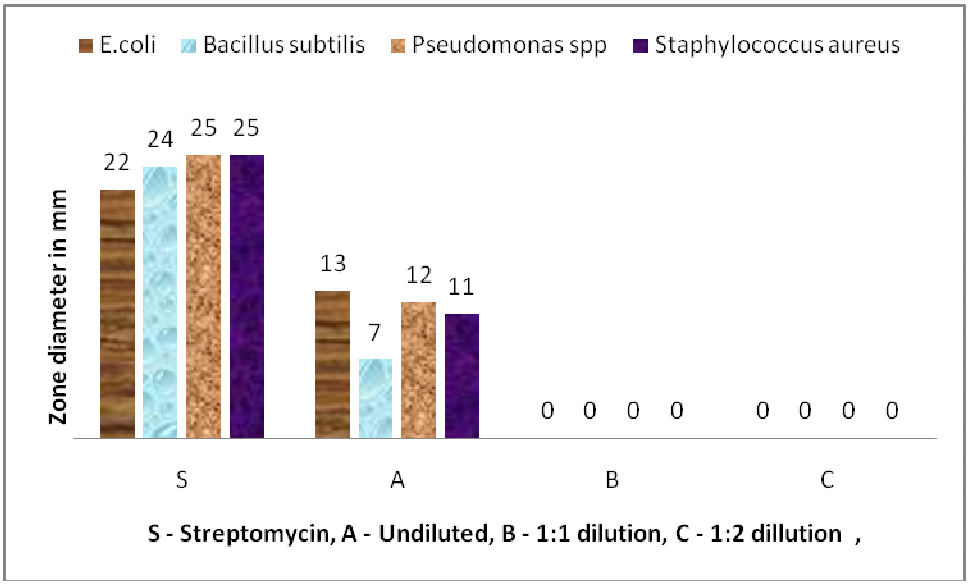

Figure 5
Antibacterial activity of crude extract of Rosmarinus officinalis

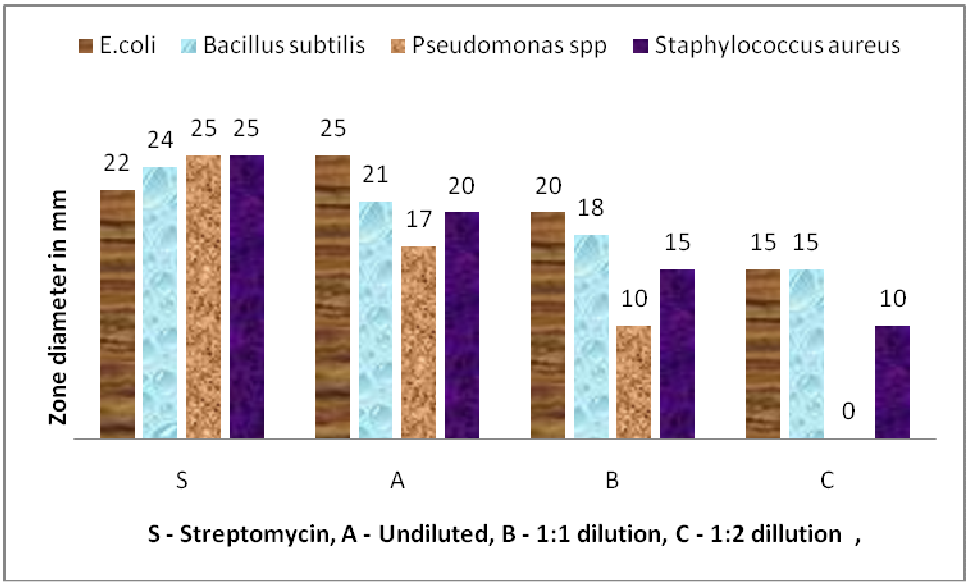

Figure 6
Antibacterial activity of essential oil of Rosmarinus officinalis

Table 2

Showing 3-way ANOVA analysis of Agar well diffusion assay of crude and essential oil extract from Rosmarinus officinalis L against bacteria

Tests of Between-Subjects Effects					
Dependent Variable: Diameter of zone of inhibition in millimeter					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Model	38370.000[a]	24	1598.750	3837.000	.000
Bacteria	994.583	3	331.528	795.667	.000
Extract	8520.417	1	8520.417	20449.000	.000
Concentration	4955.833	2	2477.917	5947.000	.000
Bacteria * Extract	1144.583	3	381.528	915.667	.000
Bacteria * Concentration	344.167	6	57.361	137.667	.000
Extract * Concentration	440.833	2	220.417	529.000	.000
Bacteria * Extract * Concentration	119.167	6	19.861	47.667	.000
Error	90.000	216	.417		
Total	**38460.000**	**240**			

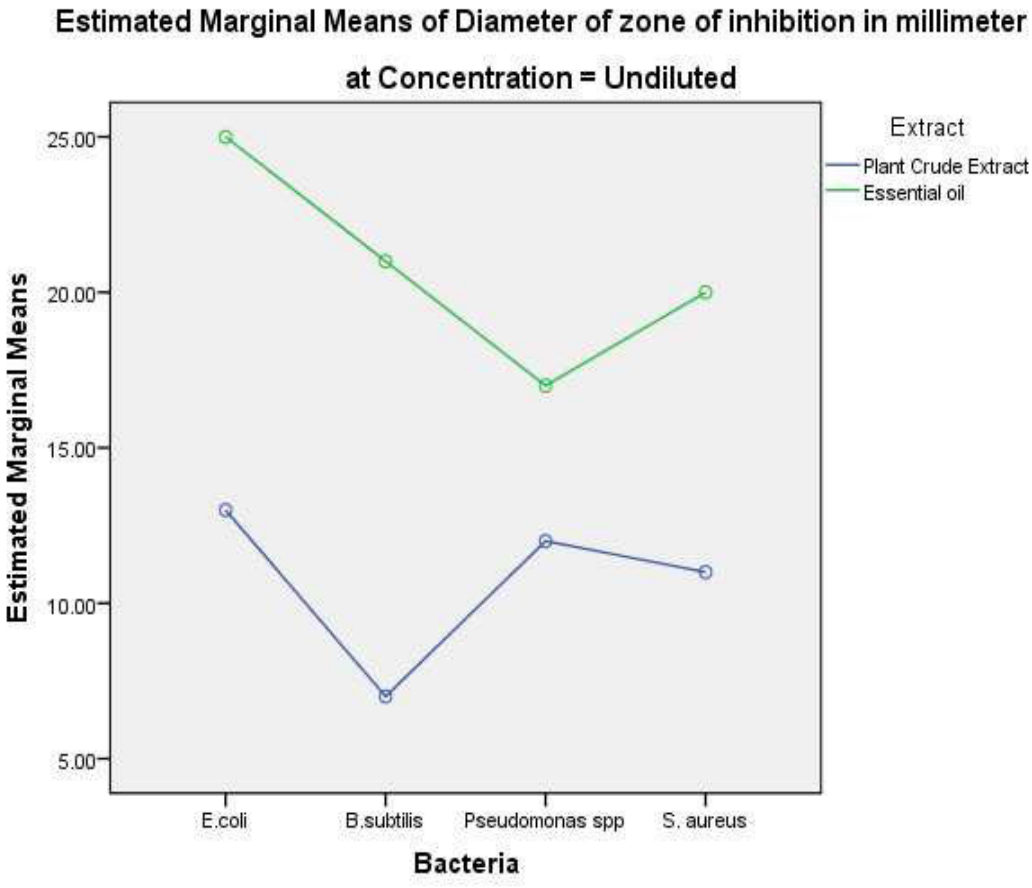

Figure 7

Comparison of the effect of crude extract and essential oil of Rosmarinus officinalis L. against Bacteria by Agar dilution method.

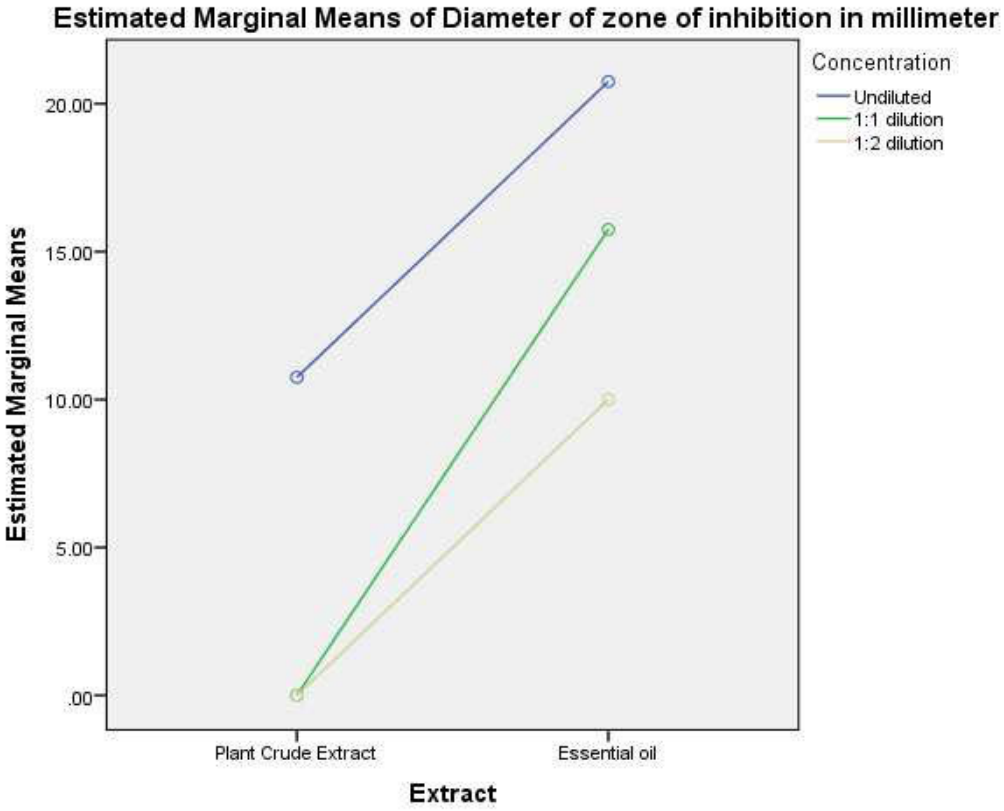

Figure 8
Comparison of the effect of different dilutions of crude extract and essential oil of Rosmarinus officinalis L

Table 3
Showing the Minimum Inhibitory Concentration (MIC) of rosemary oil from Rosmarinus officinalis L

Bacteria	MIC for Streptomycin µg/ml (Positive Control)	*In vitro* MIC for Rosemary oil (µg/ml)	Statistically calculated MIC for Rosemary oil (µg/ml)
E.coli	12.5	12.5	10.88
B. subtilis	6.25	50	42.82
Pseudomonas spp	3.125	100	*85.66*
S.aureus	6.25	50	42.82

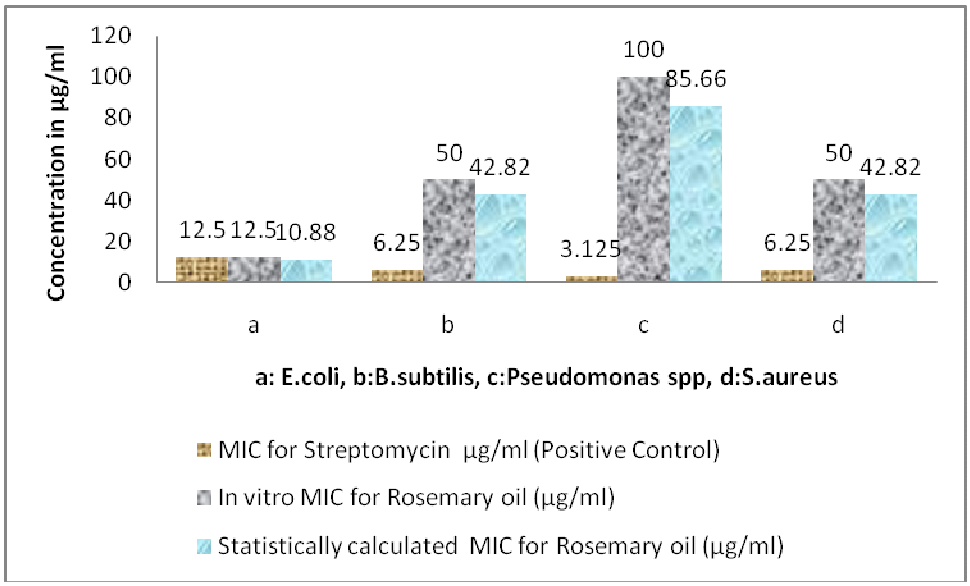

Figure 9
Minimum Inhibitory concentration (MIC) of rosemary oil

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