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IN VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF HERBAL COMBINATIONS OF *ROSEMARINUS OFFICINALIS* (ROSEMARY) AND *MENTHA PIPERITA* (PEPPERMINT)

SRIVASTAVA SN[1*] AND DIXIT D[2]

1: Amity Institute of Biotechnology, Sector-125, Amity University Uttar Pradesh, Noida-201303, India

2: Indian Agriculture Research Institute, PUSA Campus, New Delhi-110001, India

***Corresponding Author: Srivastava S. Nupur: E mail: nsinha@amity.edu**

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ABSTRACT

Herbal products are of interest to many patients and health care practitioners as about 70% of population worldwide rely on herbal medicines for part of their primary health care. When herbs are used in combination, the effects can be complicated as various interactions can occur among the individual components. The most desirable interactions are those which can result in additional therapeutic benefit. **Aim:** The present study aims at investigating the antioxidant and antimicrobial potentials of herbal combination of Rosemary and Mint extracts. This study is done to investigate the relationship between total phenolic content, antioxidant activity and antimicrobial properties of different herbal combinations. **Materials & Methods:** The antioxidant activities were determined by *in vitro* assays to compare their antioxidant effects. These include DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, FRAP (Ferric reducing antioxidant power) assay, ABTS (2,2'-azino-*bis*-3-ethylbenzthiazoline-6-sulphonic acid) assay, TFC (total flavonoid content) determination and TPC (total phenolic content) determination. The antibacterial effects of various herbal combinations of Rosemary and Mint extracts against infectious bacterial strains were also evaluated.

Results: Our results demonstrated the antioxidant potential of herbal combination of hydroalcoholic extract of Rosmary:Mint::70:30 was highest and Rosemary:Mint::10:90 was lowest. Similarly antibacterial effect was maximally exhibited by Rosemary:Mint::70:30 combination and minimally exhibited by Rosemary:Mint::10:90 using two Gram positive and Gram negative strains. Our results

suggest that hydroalcoholic extracts of Rosemary:Mint in the ratio of 70:30 has highest antioxidant potential and antibacterial property. Hence this combination is best suited for further analysis.

Keywords: Rosemary, Mint, Antioxidant, antimicrobial, hydroalcoholic extract, FRAP, DPPH

INTRODUCTION

Herb-herb combinations have been used as a medicinal practice for thousands of years, yet scientific evidence of their therapeutic benefits is lacking. With increasing interest in shifting from the one-drug-one-target paradigm to combination therapy or polypharmacy to achieve therapeutic benefits for a number of diseases, there is momentum to explore new knowledge by tapping the past empirical experiences of herb-herb combinations **[1]**. Various types of pharmacokinetic and pharmacogenomic interactions from herb-drug combinations have been well described and documented in recent literature **[2-5]**. On the other hand, much less information is available on herb-herb interaction, although herb-herb combination has been used and documented as a desirable therapeutic approach in China since the time of the Y*ellow Emperor's Canon of Internal Medicine* more than 2,000 years ago**[6]**.

A member of the mint family, Rosemary (*Rosmarinus officinalis*) is a woody herb that grows sweet-smelling, evergreen leaves and white, blue, pink, or purple flowers. Most commonly used as either a flavoring in foods or a fragrant perfume, rosemary has also been lauded in traditional medicine for its potential to help manage a variety of disorders; the principal bioactive ingredient in rosemary, rosmarinic acid, is known to possess antiviral, antibacterial, anti-inflammatory, and antioxidant activities. Rosemary herbs have also been reported to offer a long list of good health benefits, such as blood flow stimulation, stress reduction, boost to immunity, and nootropic effects. In addition to the numerous nootropic effects of rosemary exemplified in animal studies, these benefits suggest its important therapeutic role, particularly in conditions of memory loss, such as Alzheimer's, as well as general mental stimulation **[7]**.

Among several plants, *Mentha piperita* (Peppermint) is one of the herbs most widely used worldwide, with a long history of safe use in medicinal preparations. Its leaf is used as a remedy for common cold, inflammation of the mouth, pharynx, liver, as well as disorders in the gastrointestinal tract such as nausea, vomiting, diarrhea, cramps, flatulence and dyspepsia. This plant possesses polyphenols that are highly effective antioxidants and are less toxic than the synthetic ones. This property makes it of great interest to the Food

Industry, since the phenolic compounds retard the oxidative degradation of lipids improving the quality and nutritional value of food **[8]**. According to one study menthol is one of the main components of the essential oil of *Mentha piperita* that produce anti-cancer activity inducing cell death, either by necrosis or apoptosis (in Caco-2 cell line) **[9]**. The cytotoxicity associated with essential oil has been attributed to various effects such as the production of reactive species, change in fluidity and membrane permeability, tubulin polymerization, imbalance in ion transport, and inhibition of protein function **[10, 11]**. It is also used as antioxidant, antimicrobial, antiviral, anti-inflammatory, and anti-carcinogenic **[12, 13]**.

The present study was undertaken to determine ROS scavenging inhibitory activity and phytochemical content of different herbal combinations of Rosemary and Mint. This study aims at investigating the antimicrobial and antioxidant potentials of herbal combination of Rosemary and Mint extracts.

MATERIALS AND METHODS

All the reagents and chemicals used in the experiments were of analytical grade. The chemicals DPPH, TPTZ (2,4,6-tripyridyl-*S*-triazine), ABTS, 2-thiobarbituric acid, ascorbic acid and Folin-Ciocalteu reagent, were obtained from Sigma Chemical Co., USA. 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) and Trolox (6-hydroxy-2,5,7,8-tetramethyl chromane 2-carboxylic acid) were obtained from Aldrich Chemicals Co., U.S.A. All other chemicals used were obtained from the local suppliers.

1. Collection of Plant material and preparation of hydroalcoholic extracts: The leaves of *Rosemarinus officinalis* (Rosemary) and *Mentha piperita* (Peppermint) were collected from IARI, Pusa New Delhi. They were washed with distilled water to clean the adhering dust particles. The leaves were dried and grinded using mechanical grinder to fine powder. The powdered leaves were extracted twice with 70% ethanol for 24 h at room temperature. The extract was filtered through Whatman No.2 filter paper and was evaporated under the vacuum at 40°C and then it was dried to a powder using a freeze-dryer at-50°C.

Various herbal combinations were prepared of both the extracts

(i) Rosemary:Mint::50:50,
(ii) Rosemary:Mint::40:60
(iii) Rosemary:Mint::30:70
(iv) Rosemary:Mint::20:80
(v) Rosemary:Mint::10:90
(vi) Rosemary:Mint::60:40
(vii) Rosemary:Mint::70:30
(viii) Rosemary:Mint::80:20
(ix) Rosemary:Mint::90:10

2. Antioxidant profiling of hydroalcoholic extracts:

(i) DPPH assay: DPPH is a stable free-radical molecule widely used assay to measure the free radical scavenging capacity of plant extracts. Assay was initiated by adding 200 µl of 0.004% DPPH methanolic solution into 96-well plate, followed by addition of 20 µl of *extract*, or solvent or the blank. The mixture was incubated at 30ºC for 1 h and the absorbance was measured at 517 nm in a microplate reader. The DPPH scavenging activity (%) was calculated based on the following formula:DPPH scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$ where A_0 is absorbance of blank, A_1 is absorbance of test sample. A calibration curve was plotted with % DPPH scavenged versus concentration of standard antioxidant (Trolox) **[14]**.

(ii) FRAP assay: FRAP (Ferric reducing antioxidant power) assay is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+}-tripyridyltriazine (blue colored complex) formed by the action of electron donating antioxidants at low pH. This reaction was monitored by measuring the change in absorbance at 593 nm. The FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 ml TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37°C. Freshly prepared working FRAP reagent was pipetted using 1-5 ml variable micropipette (3.995 ml) and mixed with 5 µl of the appropriately diluted plant sample and mixed thoroughly. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml FRAP reagent+5 µl distilled water) after 30 min incubation at 37°C. All the determinations were performed in triplicates. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$. The concentrations of $FeSO_4$ were in turn plotted against concentration of standard antioxidant trolox. The FRAP values were obtained by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mg of Trolox equivalent per gram of sample **[15]**.

(iii) ABTS assay: Free radical scavenging activity of plant samples was determined by ABTS radical cation decolorization assay. ABTS cation radical was produced by the reaction between 7mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. ABTS + solution was then

diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After the addition of 5 µl of plant extract to 3.995 ml of diluted ABTS + solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent inhibition of absorbance at 734 nm was calculated using the formula, ABTS·+ scavenging effect (%) = $((A_0–A_1)/ A_0)\times100$ where A_0 absorbance of ABTS radical + methanol; A_1 is absorbance of ABTS radical + sample extract/standard Trolox was used as standard substance**[16]**.

(iv) TFC (total flavonoid content) determination: The $AlCl_3$ method was used for quantification of the total flavonoid content of the methanolic plant extracts. 20 µl of the sample extract was added to a solution of 2% $AlCl_3$.6H2O. The mixture was vigorously shaken and diluted with double distilled water to make the total volume 10 ml. The absorbance of the reaction mixture was measured after 10 min incubation at 440 nm. Flavonoid contents were expressed as quercetin equivalents in mg per gram dry material. All the determinations were performed in triplicates **[17]**.

(v) TPC (total phenolic content) determination: The total phenolic content was determined spectrophotometrically by using Gallic acid to set up the standard curve. The content of phenolic compounds of the samples was expressed as gallic acid equivalents (GAE) in mg per gram dry weight. All the samples were analyzed in triplicates **[18]**.

3. Antimicrobial studies of hydroalcoholic extracts*:* Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. The agar plate surface was inoculated by spreading a volume of the microbial inoculum (Two Gram positive; *S. aureus and B. subtilis* and two Gram negative bacteria; *E. coli and K pneumoniae*) over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration was introduced into the well. Then the Petri plates were transferred to incubation chamber for 24hr at 37°C. After incubation, antibacterial activity was determined by measuring the zone of inhibition around the well by millimeter using a transparent ruler. The measurements were performed in triplicates to determine the mean of the inhibition zone. Greater the diameter more active is plants extracts tasted on the colony of the organisms **[19, 20]**.

4. Statistical Analysis:

All the experiments were done in triplicate and the results were expressed as Mean±SD. The data were statistically analyzed using one-way ANOVA followed by Duncan's test. Mean values were considered statistically significant when $p>0.05$.

RESULTS & DISCUSSION

In the present investigation, the commonly accepted assays viz DPPH, FRAP and ABTS were used for the evaluation of antioxidant activity of plant extracts. The total phenolic contents and flavonoid contents were also determined. The free radical scavenging activity of various herbal combinations were detected by measuring the inhibition of DPPH radical by antioxidants present in the herbal combination of Rosemary and Mint extracts. The total antioxidant activity, as measured by TEAC (Trolox equivalent capacity), ranged from 0.20 to 1.54 mg trolox equivalent per g dry weight (mg, TEAC/g dw). The results from the DPPH assays showed that all the combinations of both the plants can scavenge the free radical to a certain extent **(Table 1)**. However maximum free radical scavenging activity was shown by Rosemary: Mint::70:30 combination whereas lowest scavenging activity was shown by Rosemary: Mint:: 10:90 combination **(Figure 1)**.

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+} -TPTZ). The free radical chain breaking takes place through donating a hydrogen atom. At low pH of about 3.6, reduction of Fe^{3+} -TPTZ complex to blue colored Fe^{2+} -TPTZ takes place, which has absorbance at 593 nm. FRAP values of the studied plants varied from 0.45 mg, Trolox equivalent/g dw of sample (Rosemary:Mint::10:90) to 14.75 mg, trolox equivalent/g dw of sample (Rosemary:Mint::70:30) **(Table 1)**. The highest reducing power was shown by Rosemary:Mint ::70:30 combination whereas lowest reducing power was shown by Rosemary:Mint ::10:90 combination **(Figure 2)**. The results obtained are highly reproducible and are in accordance with the results already reported **[21, 22]**.

The relative antioxidant ability to scavenge the radical $ABTS^{+}$ has been compared with the standard Trolox. ABTS radical cation was produced in the stable form using potassium persulphate. After getting the stable absorbance, various combinations of both the plant extracts is added to the reaction medium and the antioxidant power was measured by studying decolorization.

The TEAC values ranged from 0.62 to 7.41 mg, TEAC/g dw **(Table 1)**. The lowest value (0.62 mg, TEAC/g dw) was observed for combination Rosemary:Mint::10:90 and highest value (7.41 mg, TEAC/g dw) for combination Rosemary:Mint::70:30 was observed **(Figure 3)**.

a) TEAC, Trolox equivalent capacity (mg Trolox equivalents/gdw). Absorbance was converted to equivalent activity of Trolox per g of dry weight based on a standard curve.
b) Total phenolic content expressed as mg of Gallic acid equivalent (GAE)/g of dry weight (dw).
c) Total flavonoid content expressed as mg of quercetin equivalents/g of dry weight (dw).

It is observed from **Table 1** that the total phenolic content of the samples ranges from 0.48 to 12.85 mg Gallic acid equivalent/g dw. The combination Rosemary:Mint::70:30 showed the highest amount of phenolic content (12.85 mg, GAE/g dw) while the lowest content was observed in combination Rosemary: Mint::10:90 (0.48 mg, GAE/g dw) **(Figure 4)**. The considerable difference between the results of phenolic content are may be due to environmental related factors like maturity period, climate, location, temperature, fertility, diseases, part tested and pest exposure **[23]**. In addition, rainfalls are also reported to affect the phenolic content **[24]**. The presence of different phenolic compounds may be the cause in the variation of total phenolics in the plant extracts.

It is observed that almost all the combinations of Rosemary and Mint were rich in flavonoids. The total flavonoid content is found to vary between 1.24 (combination Rosemary:Mint::10:90) to 13.87 mg quercetin equivalent/g dw (combination Rosemary:Mint::70:30) as shown in **Figure 5**. The compounds such as flavonoids and polyphenols which contain hydroxyls are responsible for scavenging effect of plants.

It was also observed that the phenolic and flavonoid contents correlates well with DPPH, FRAP and ABTS assays. The combinations of Rosemary and Mint (R:M::70:30) which showed highest phenolic and flavanoid contents, also showed highest antioxidant activities as measured by DPPH, FRAP and ABTS assays. The combinations of Rosemary and Mint (R: M :: 10:90) which showed lowest phenolic and flavanoid contents, also showed lowest antioxidant activities as measured by DPPH, FRAP and ABTS assays. Our results agree with the findings in which a strong correlation between total phenolic content and FRAP assay has been reported **[25]**. It could be estimated that the

phenolic compounds present in the extracts act as an antioxidant directly through the mechanism of the reduction of oxidized intermediate in the chain reaction.

Various combinations of hydroalcoholic extracts of Rosemary and Mint were subjected to a preliminary screening for antimicrobial activity against Gram positive bacteria i.e. *Bacillus subtilis*, *Staphylococcus aureus* and Gram-negative bacteria i.e. *Escherichia coli and Klebsiella pneumoniae*. It was clear from **Table 2** that the combination Rosemary:Mint::70:30 showed significant activity against four organisms tested but the combination Rosemary:Mint::10:90 showed very less activity against both Gram positive and Gram negative bacteria as compared to the standards drug 25 µg/ml Gentamicin against all organisms.

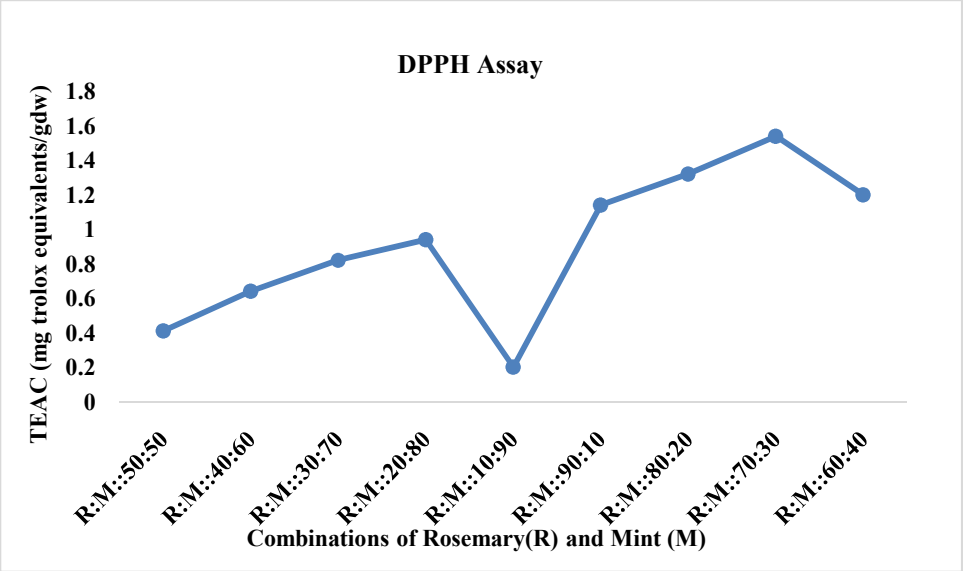

Figure 1

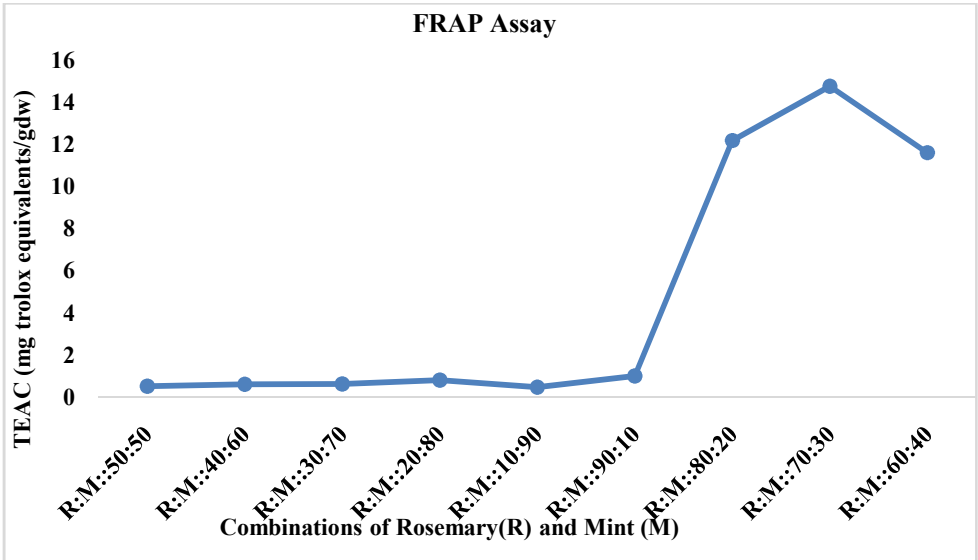

Figure 2

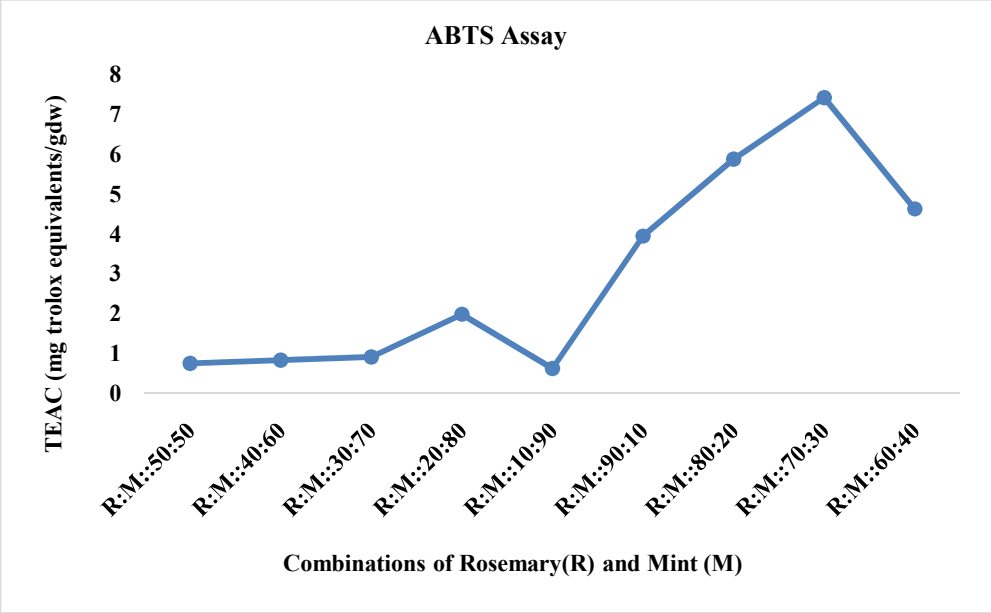

Figure 3

Table 1: Antioxidant capacity, total phenolic and flavonoid content values of different combinations of Rosemary and Mint

Combinations of Rosemary and Mint	DPPH	FRAP	ABTS	Total Phenolic Content	Total Flavanoid Content
	TEAC, Trolox equivalent capacity (mg trolox equivalents/gdw)			(mg GAE/gdw)	(mg quercetin/gdw)
Rosemary:Mint::50:50	0.41±0.03	0.50±0.04	0.75±0.08	0.61±0.23	2.17±0.08
Rosemary:Mint::40:60	0.64±0.09	0.59±0.09	0.83±0.01	0.83±0.96	3.49±0.12
Rosemary:Mint::30:70	0.82±0.10	0.6 1±0.08	0.91±0.03	2.92±0.19	5.39±0.23
Rosemary:Mint::20:80	0.94±0.09	0.79±0.10	1.98±0.11	5.27±0.86	7.43±0.58
Rosemary:Mint::10:90	0.20±0.03	0.45±0.02	0.62±0.07	0.48±0.39	1.24±0.35
Rosemary:Mint::90:10	1.14±0.06	0.98±0.13	3.94±0.08	7.12±0.16	9.85±0.56
Rosemary:Mint::80:20	1.32±0.11	12.17±0.24	5.87±0.19	10.27±0.21	11.45±0.52
Rosemary:Mint::70:30	1.54±0.15	14.75±0.45	7.41±0.20	12.85±0.52	13.87±0.68
Rosemary:Mint::60:40	1.20±0.11	11.59±0.16	4.62±0.09	9.15±0.76	10.38±0.42

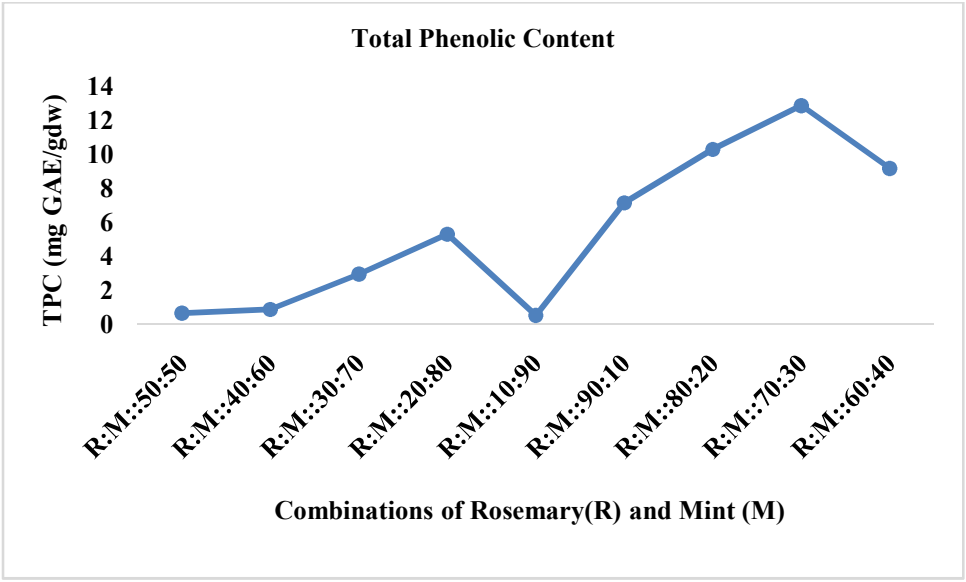

Figure 4

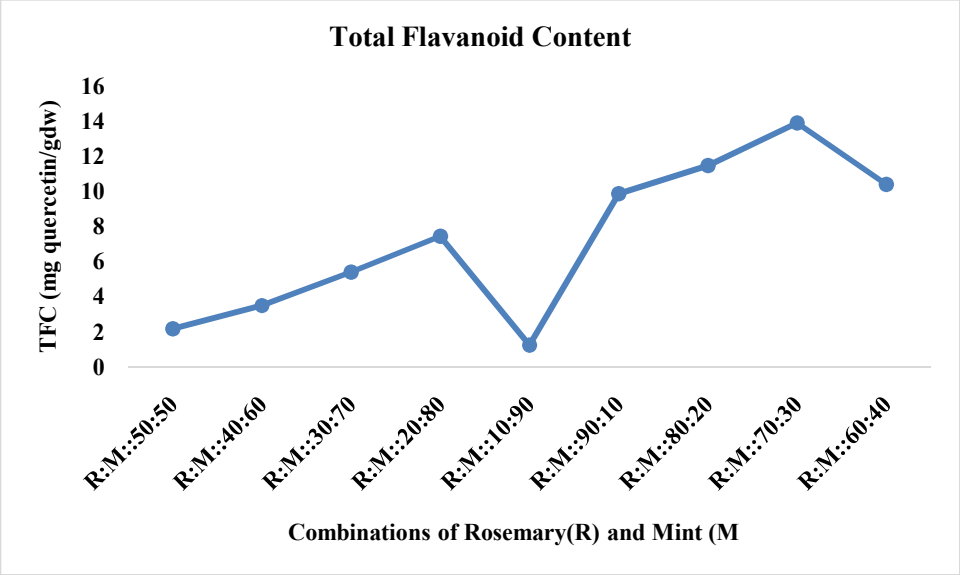

Figure 5

Table 2: Antibacterial effects of different combinations of Rosemary and Mint

Test Samples	Zone of Inhibition (mm)			
	Gram positive Bacteria		Gram negative Bacteria	
	S. aureus	*B. subtilis*	*E. coli*	*K. pneumoniae*
Rosemary:Mint::50:50	11	18	12	13
Rosemary:Mint::40:60	13	13	14	11
Rosemary:Mint::30:70	12	17	13	10
Rosemary:Mint::20:80	14	15	16	12
Rosemary:Mint::10:90	10	12	14	11
Rosemary:Mint::90:10	16	21	17	13
Rosemary:Mint::80:20	15	21	18	14
Rosemary:Mint::70:30	17	23	21	16
Rosemary:Mint::60:40	16	20	18	15
Gentamicin (25 µg/ml)	18	25	22	17

CONCLUSION

The present study provides the useful information about antioxidant properties and antimicrobial effects of various combinations of Indian medicinal plants, Rosemary and Mint which are used for the therapeutic purposes. It is interesting that the total phenolic content and flavonoid content correlate well with the results from the DPPH, FRAP and ABTS tests. The findings of this study support the fact that some medicinal plant extracts which are commonly consumed in India, if used in combination, can be a promising source of potential antioxidants and antimicrobial agents.

For thousands of years, natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs **[26]**. In the present work, an attempt has been made to check various combinations of two medicinal plants extracts, Rosemary and Mint for its antioxidant and antibacterial activities. Since, medicinal plants constitute a wide variety and diversity of secondary

metabolites, medicinal plants could be used as a good source of antibacterial agents **[27]**. For instance, bioactive metabolites such as tannins and alkaloids **[28]**, polyphenolic biomolecules **[29]** and flavonoids **[30, 31]** have been found to have antimicrobial properties. The presence of such metabolites in the studied plant extracts can provide a preliminary explanation on their antibacterial activities. Further investigations are required to identify their active metabolites.

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Conflict of interest

The authors declare that they have no conflict of interests

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