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A Study on Combinatorial Effects of Various Flavonoids for Their Antibacterial Potential Against Clinically Significant Bacterial Species

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Abstract

In the present study, combinatorial effects of various flavonoids were evaluated against two Gram-positive and two Gram-negative bacteria of clinical relevance. The results indicated the antibacterial potential of each flavonoid under mild-to-moderate concentrations; however, their effectiveness increased when both flavonoids were used in combination with various degree of concentrations. It has also been observed that Gram-positive bacteria were found more susceptible than that of Gram-negative bacteria; the reasons can be attributed towards their cell wall structure. Flavonoids and their optimal combinations could be used as suitable alternatives towards finding new antimicrobial for the treatment of infectious diseases.

INTRODUCTION

Flavonoids are polyphenolic compounds that are categorized according to their chemical structure *viz*. flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. They are secondary metabolites that have no direct involvement with the growth or development of plants. More than 4000 flavonoids have been identified so far, many of which occur in fruits, vegetables and beverages such as tea, coffee, beer, wine and fruit drinks.

The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Several plant spices containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine [1]. They have been reported to have antiviral [2,3], antimicrobial [4], anti-allergic [5], antiplatelet, anti-inflammatory [6], antitumor [7] and antioxidant activities [8]. Flavonoids biologically trigger the production of natural enzymes that fight disease. Flavonoids are also proved to be potential aromatase inhibitors [9].

The current study dealt with the comparative analysis of antibacterial potential of different combinations of some flavonoids against a number of clicinically significant bacterial species. This study is very necessary in order to determine the sensitivity of these bacterial species which are developing resistance against currently available antibiotics and are very difficult to treat. Thus novel

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sources of chemotherapeutic agents should be discovered and natural resources such as flavonoids are gaining increased significance in this context.

MATERIALS AND METHODS

Compounds: High purity flavonoids (99% purity) i.e. quercetin (3,5,7,3',4'-pentahydroxyflavone) and morin (3,5,7,2',4'-pentahydroxyflavone) were purchased from Hi-Media, Mumbai, India for the current study.

Microorganisms: The bacterial strains selected for the current study were Gram-negative (*Escherichia coli* MTCC-739 and *Salmonella typhi* MTCC-531) and Gram-positive (*Bacillus subtilis* MTCC-736 and *Staphylococcus aureus* MTCC-740). The bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the microorganisms was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

Bacterial susceptibility assay and determination of MIC: A 24 h culture of each bacterial strain was transferred to 30 ml of Mueller-Hinton broth (Hi-Media) and incubated at 37ºC on shaker incubator at 150 rpm for 20 h. These cultures were then used as inocula for the study. Minimum inhibitory concentrations (MIC's) required for the bacteriostatic effects were determined by standard two-fold broth microdilution methodology [10]. A stock solution of flavonoid was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 1.0 μg/ml to 512 μg/ml. A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately $5x10^5$ CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the sample inhibiting the visible growth of bacterial strain using reflective viewer.

RESULTS AND DISCUSSION

In the recent years, flavonoid such as chalcones, flavanones and flavones have gained increasing interest due to their numerous applications and properties [11]. Thus, an attempt has been made in the current study to evaluate the flavanoids for their comparative antibacterial potential against various clinically significant bacteria.

Flavonoids (quercetin and morin) were used in concentration ranging from 1.0 μg/ml to 512 μg/ml. When tested separately; quercetin demonstrated the inhibition of Gram-negative *Salmonella typhi* and *Escherichia coli* at 128 and 64 μg/ml concentration, respectively; however, morin shows the equal inhibition of both bacteria. Gram-positive bacteria are inhibited by quercetin at 64 and 32 μg/ml concentration, respectively for *Bacillus subtilis* and *Staphylococcus aureus*. Morin was found to have similar effect against both bacteria. Table 1 demonstrates the comparative analysis of each of the flavonoids and their combination for their respective potential against selected microorganism. Both of the flavonoids were further evaluated in combination to study their synergistic effect. Furthermore, both flavonoids were found almost equal inhibitors against Gram-negative *Salmonella typhi* and *Escherichia coli*, however; their combination significantly reduces the growth of *Escherichia coli* at 32 μg/ml concentration. Their inhibitory effect, in case of Gram-positive bacteria were found imbalanced as *Staphylococcus aureus* was inhibited greatly that that of *Bacillus subtilis*. Combination of flavonoids showed the static effect on the growth of both of the bacterial species with 16 μg/ml and 32 μg/ml concentrations, respectively.

Quercetin was found comparatively more effective than that of morin; however; differences in MICs for both flavonoids were not much significant when used them separately. These results were found to be slightly contradictory to that of the work done by Alvarez et al. [12]; which may be attributed possibly due to different methodology used in pursing the work.

The present work greatly elucidates the antibacterial potential of flavonoids. It has also been confirmed that the combination of both flavonoids cause evident reduction of MICs against selected microorganisms, however; efficacy was found higher against Gram-positive bacteria than that of Gram-negative bacteria.

A number of research works has been carried out to investigate the antibacterial activity of numerous flavones and their derivatives against *Staphylococcus aureus* and *Escherichia coli* [11,13]. Previous research works were emphasized only against a few common bacteria, but we have extended our research work with clinically relevant bacterial species. Efficacy of flavonoids was found to be significant against Gram-positive bacteria in comparison to Gram-negative bacteria. These results can be attributed to the cell-wall structure of both type of bacterial cells. In Gram-negative bacteria, presence of lipopolysaccharide layer outside the peptidoglycan layer makes cell wall more complex and renders it to almost impermeable for entry of foreign molecules. However, Gram-positive bacteria do have only peptidoglycans in their cell wall. This will help in an easy penetration of the molecules inside the cell; thus shows the greater sensitivity [14].

The combinatorial or synergistic effect of these flavonoids could be an effective alternative in the treatment of various pathogenic infections.

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Table 1. Minimum Inhibitory Concentrations (MIC's) of selected flavonoids and their combinations

		Concentration of Compound (in µg/ml)										
Compound	Microorganism	512	256	128	64	32	16	8	4	2	1	MIC (in µg/ml)
Quercetin	*Salmonella typhi*	-	-	-	+	+	+	+	+	+	+	128
Morin	*Salmonella typhi*	-	-	-	+	+	+	+	+	+	+	128
Quercetin + Morin	*Salmonella typhi*	-	-	-	-	+	+	+	+	+	+	64
Quercetin	*Escherichia coli*	-	-	-	-	+	+	+	+	+	+	64
Morin	*Escherichia coli*	-	-	-	+	+	+	+	+	+	+	128
Quercetin + Morin	*Escherichia coli*	-	-	-	-	-	+	+	+	+	+	32
Quercetin	*Bacillus subtilis*	-	-	-	-	+	+	+	+	+	+	64
Morin	*Bacillus subtilis*	-	-	-	-	+	+	+	+	+	+	64
Quercetin + Morin	*Bacillus subtilis*	-	-	-	-	-	+	+	+	+	+	32
Quercetin	*Staphylococcus aureus*	-	-	-	-	-	+	+	+	+	+	32
Morin	*Staphylococcus aureus*	-	-	-	-	+	+	+	+	+	+	64
Quercetin + Morin	*Staphylococcus aureus*	-	-	-	-	-	-	+	+	+	+	16

(-) represents 'No Growth Observed'; (+) represents 'Growth Observed'.