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Original Article

STUDY OF SYNERGISTIC EFFECTS ON ANTIOXIDANT ACTIVITY AND ANTIMICROBIAL ACTIVITY OF POLYHERBAL FORMULATIONS CONTAINING FICUS SPECIES

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ABSTRACT

Objective: The present study aims at screening the synergistic effect on the therapeutic efficiency of traditional herbal medicine *Nalpamaradi Choorna* and *Nalpamaradi Keram,* containing four *Ficus* species. The efficiency of formulations prepared by mixing crude drug is tested concerning their Antioxidant and Antibacterial activities. It will also provide and validate the use of these drugs in the current trend of targeted Combination Therapy for various neurodegenerative diseases.

Methods: The in-vitro studies of the methanol extracts of the barks of the four individual plants, their different combinations, and the *choorna* were conducted by DPPH method, and the obtained EC_{50} values were compared to evaluate the synergistic effect. The antibacterial activity of *Nalpamaradi Keram* and the four *Ficus* plants was tested against two microorganisms *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* using agar well method.

Results: The formulation prepared by mixing equal proportions of the four plants exhibited maximum %AA value of 93.36 % and EC_{50} value 8.00 µg/ml while the marketed drug showed maximum % AA of 87.30 % and an EC_{50} value of 29.0µg/ml. The combination of *Ficus bengalensis (F. ben), Ficus racemosa (F. rac) and Ficus religiosa (F. rel)* demonstrated the maximum % AA of 92.35 % and a very low EC_{50} value of 6.00µg/ml. All the samples except *Ficus microcarpa (F. mic)* exhibited antibacterial property against both the bacteria. *Nalpamaradi Keram* has shown the zone of inhibition of 20.0 mm against *S. aureus* and 18.0 mm against *E. coli.*

Conclusion: The present investigation justifies the traditional use of these medicinal plants as antibacterial and antioxidant agents and validates their synergistic effect with improved activity in the formulations. Therefore, it is judicious to mix all the four *Ficus* species in the formulation of *Nalpamaradi choorna* and *Nalpamaradi keram.*

Keywords: Antioxidant and synergistic activity, Antimicrobial activity, Ficus species, Polyherbal formulations, Radio protectivity

INTRODUCTION

The present studies aim at screening the therapeutic efficiency of traditional medicines based on purely natural products than discovering a new drug which requires more investments and greater challenges. The effectiveness of formulations prepared by mixing crude drugs is tested concerning their pharmacological activities [1].

The genus *Ficus* constitutes an important group of trees with immense medicinal values. Among the many species, four trees, i.e., *F. benghalensis (F. ben), F. racemosa (F. rac), F. religiosa (F. rel) and F. microcarpa (F. mic)* form a group '*Nalpamara*' in Ayurveda. The bark leaves, and fruits of these *Ficus* species are used as astringent, haemostatic, antiseptic, anti-inflammatory, antioxidant and anticancer agents. They are also utilized in the treatment of diarrhea, dysentery, skin diseases, ulcers, vaginal disorders, leucorrhoea, menorrhagia, and deficient lactation [2]. *Ficus* species are reported as a rich source of naturally occurring antioxidants of which phenolic compounds and flavonoids play a vital role in preventing numerous health disorders related to oxidative stress including cardiovascular diseases, neurodegenerative diseases, and cancer [3].

The barks of these trees are important ingredients of many Ayurvedic formulations like *Nalpamaradi Choorna, Nalpamaradi Keram, Saribadyasavam, Chandanasavam,* etc.

In the present study, we aimed at the evaluation of the antioxidant activity of these four *Ficus* species and *Nalpamaradi Choorna*, a formulation made out of them. The synergistic effect of the antioxidant activity of these four constituents was studied by mixing them in different combinations. The antibacterial activity of these four plants and an oil formulation, *Nalpamaradi Keram,* was tested against two microorganisms *S. aureus* (gram positive) and *E. coli* (gram-negative).

MATERIALS AND METHODS

DPPH (2, 2-Diphenyl-1-picryl hydrazyl radical) was purchased from Sigma-Aldrich. Methanol was from Merck India. U. V-Visible spectrophotometer (Elico-India SL-207) was used for the analysis. The tested bacteria cultures were obtained and authenticated from the Department of Microbiology, K. J. Somaiya College. Vidyavihar, Mumbai, India. Fresh barks of the four *Ficus* plants were collected from Somaiya Vidyavihar Campus, Mumbai, India, in the month of March-April 2015 and authenticated by Blatter Herbarium, St. Xavier's College, and Mumbai. *Nalpamaradi Choorna* and *Nalpamaradi Keram* were procured from a local Ayurvedic Pharmacy.

Bark powders (10 g each) of F. ben*, F. rac, F. rel, and F. mic* and the formulations were extracted with 4:1 methanol: water in a 200 ml Soxhlet extractor for 24 h. The solvent was removed by using a rota-evaporator. The methanol extracts yield a dark brownish solid residue.

Antioxidant assay

All the samples were dissolved in 95 % methanol to obtain 10 µg/ml solutions and diluted subsequently for the antioxidant assay. Ascorbic Acid was used as a standard for comparison in all assays done in triplicate. DPPH radical scavenging activity assay was carried out according to the method of Brand-Williams, Cavalier, and Berset with some modifications. This assay provides information on the reactivity of test compound with a free radical since odd electron of DPPH gives strong absorption band at 517 nm (violet colour) and when it is quenched by the extract, there is a decrease in

absorbance. The percentage Antioxidant Activity (% AA) was measured by the formula.

$$\% AA = (A_c - A_t) \times 100 / A_c.$$

A_c is the absorbance of Standard; A_t is the absorbance of the test sample.

EC50 value is the effective concentration that could scavenge 50 % of the DPPH radicals.

Agar well diffusion assay

The antimicrobial activity was measured by Agar Well Diffusion assay [4]. The plant extracts were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Petri plates containing 20 ml of the medium were seeded with the bacterial strains. Each labeled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. Wells were punctured, and 100 µl of the methanol bark extracts were added. The plates were then incubated at 37 °C for 24 h. All samples were tested in triplicates. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well in millimeters. [5]. Methanol was used as the control, and Benzyl Penicillin was the standard.

RESULTS AND DISCUSSION

Free radical scavenging activity

Free radicals are defined as any species that contains one or more unpaired electron and capable of independent existence. These active by-products are reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that come from cellular redox processes [6]. Reactive oxygen species include superoxide (O_2^{*-}), thiyl (RS^*), trichloroethyl (CCl_3^*) or nitric oxide (NO^*) radical in which the unpaired electron is delocalized between both atoms. The O_2^{*-}, hydroxyl radical ($^*OH$) and other reactive oxygen species such as H_2O_2, ozone, etc. which form free radicals in tissues through various chemical reactions are continuously produced in cells [7]. At low or moderate levels, these compounds exert a beneficial effect on cellular responses and immune functions. However, at higher concentrations, they generate oxidative stress that can damage the cell structures [8, 9]. Thus, it plays a dual role i.e. toxic and beneficial to health.

An antioxidant is a molecule that inhibits the oxidation of other molecules. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play a significant role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxide. The free radical scavenging action is considered to be one among the various methods for investigating the antioxidant activity.

The free radical scavenging activity of the crude drugs and formulations prepared using them was studied by a spectro-photometric method based on the reduction of DPPH.

In this method, DPPH is reduced in presence of hydrogen-donating antioxidant (AH) due to the formation of non-radical form DPPH-H

DPPH+AH →DPPH-H+A

In the living system, several physiological functions are modulated with the help of free radicals of different forms generated in cells. A certain level of free radicals is essential for good health as they are involved in fighting infection and the contraction of smooth muscles in the blood vessels. The body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of reactive oxygen species [10, 11]; but continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated [12]. The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their toxic effects and contribute to disease prevention.

In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity [13-16]. Endogenous and exogenous antioxidants act like separate species and enhance the immune defense while also lowering the risk of cancer and degenerative diseases [17]. Antioxidants neutralize free radicals before they attack healthy cells [18]. However, if produced in excess, they can be destructive leading to inflammation, ischemia, lung damage and other degenerative diseases [19].

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [20]. Phenolic compounds contribute to the quality and nutritional value regarding modifying color, taste, aroma, and flavor and also in providing health benefits effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores [21].

Optimization of the concentration of antioxidant compound

The antioxidant activity of all the samples was studied against DPPH solution. The %AA value was found to increase with increasing concentration of all the samples and the standard. Obviously, the scavenging effect of the samples on DPPH also increased with increase in concentration. The maximum radical scavenging or antioxidant activity was demonstrated by *F. ben* (93.36 % at 30.0 µg/ml), followed by *F. mic* (93.12 % at 40.00 µg/ml), *F. rac* (83.61 % at 50.00 µg/ml), and *F. rel* (81.00 % at 50.00 µg/ml) as against Ascorbic Acid (93.35 % at 50.00 µg/ml. [fig.1][table. 1] Thus the antioxidant activity of the four ingredients in the formulation increased in the following order *F. ben > F. mic > F. rac > F. rel* when compared with the standard Ascorbic acid.

Table 1: Variation of antioxidant activity with concentration

S. No.	Concentration (µg/ml)	*F. ben* %AA	*F. rac* %AA	*F. rel* %AA	*F. mic* %AA	Ascorbic acid %AA
1	5.**0**	25.26±0.02	9.08±0.021	33.24±0.015	31.2210.007	30.88±0.03
2	10.0	38.87±0.03	34.00±0.01	39.82±0.02	51.68±0.04	34.22±0.01
3	15.0	56.48±0.0	44.30±0.006	49.04±0.03	62.37±0.02	39.87±0.02
4	20.0	77.08±0.02	49.95±0.009	54.25±0.008	70.46±0.01	46.62±0.008
5	25.0	81.95±0.01	58.91±0.008	62.46±0.007	80.84±0.01	61.02±0.03
6	30.0	93.13±0.02	64.78±0.03	68.68±0.02	88.40±0.008	71.21±0.009
7	40.0	93.02±0.03	74.09±0.01	75.16±0.01	93.13±0.01	88.26±0.01
8	50.0	93.02±0.02	83.61±0.02	81.00±0.01	93.21±0.003	93.35±0.02

mean±SD, n= 3.

Antibacterial activity

The aqueous extract of *F. rel* is reported to have high antimicrobial activity against *Bacillus subtilis* (*B. subtilis)* and *Pseudomonas aeruginosa (P. aeruginosa)* (multi-drug resistant) [22]. The ethanol extracts of leaves at 25 mg/ml was active against *B. subtilis, S. aureus, E. coli* and *P. aeruginosa* [23] whereas fruit extract is reported to have high antibacterial activity [24].

The aqueous or alcoholic extracts of various parts of *F. ben* were found to have antibacterial activity [25]. The bark extracts of *F. rac* showed moderate antibacterial activity against *P. aeruginosa, E. coli, Proteus vulgaris (P. vulgaris), B. subtilis* and *S. aureus* [26]. Methanol extracts of bark, fruits and leaves of *F. mic* exhibited excellent antioxidant activities and possessed antibacterial activity against gram-positive and gram-negative bacteria [27].

The antibacterial activities of medicinal plants are attributed to the presence of flavonoids, tannins and steroidal alkaloids [28].

Methanol extracts of the barks of all the four *Ficus* species namely *F. ben, F. rac, F. rel,* and *F. mic* were studied for their antibacterial activity against two bacterial species (*S. aureus* and *E. coli*). *F. ben, F. rac, and F. rel* was found to be more active against *S. aureus* (gram positive) than *E. coli* while *F. mic* was not effective. The zone of inhibition was found to be the maximum for *F. rel* (18.0 mm) and minimum for *F. ben* (14.0 mm) while *F. rac* had moderate activity (15.0 mm). Thus the activity increases in the order, *F. mic < F. ben<F. rac<F. rel.*

Table 2: Antibacterial activity (+++High,+Slight,-Negative.)

Sample	*F. ben*	*F. rac*	*F. rel*	*F. mic*	Combination	*Nalpamaradi. keram*	Benzyl Penicillin (0.6µg/ml)
E. coli	+	+	+	-	+	+	-
S. aureus	+++	+++	+++	-	+++	+++	+++
Zone of Inhibition in mm (S. aureus)	14.0±0.2.	15.0±0.4	18.0±0.3	-	19.0±0.2	20.0±0.5	20.0±0.5

Control: 95 % Methanol, Standard; Benzyl Penicillin; mean±SD, n= 3.

Synergistic effect

The increasing evidence has shown that multiple active component combinations of the crude materials could amplify the therapeutic efficacy of each agent, representing a new trend in modern medicine [29, 30]. However, the precise mechanism of synergistic action remains poorly understood. In order to provide some evidence for the synergistic action, marketed drug having a mixture of all the four ingredients is compared to the amplification of antioxidant activity and antibacterial activity with that of the isolated components and their various combinations.

The various combinations showed enhanced antioxidant activity. When *F, race* was mixed with *F. ben,* the EC_{50} value became 11.0 µg/ml which was better than the individual ones, i.e., 13.0 and 20.0 µg/ml. This value was further improved by the addition of *F. rel* as it became 6.0 µg/ml. This combination demonstrated the best synergy as the maximum activity was (92.33 %) obtained at just 20 µg/ml. For *F. rel* alone it was 16.0 µg/ml. When *F. mic* was also added to this mixture, a decrease in the property is observed. i.e. 8.0 µg/ml and the maximum %AA are observed at 25.0 µg/ml. Even though *F. mic* was the best among the individual ones (9.0 µg/ml), it was not contributing to the synergy. At this juncture, it is noteworthy to observe that *F. mic* has not responded to the two microorganisms that we have studied. Still this prepared laboratory formulation was much better than the marketed drug as it exhibited maximum activity at 8.0 µg/ml against 29.0 µg/ml (max. %AA 87.30 at 50.0 µg/ml).

A combination of the four was found to be more effective against *S. aureus* than *E. coli* (16.0 mm.). A commercially available formulation, *Nalpamaradi Keram*, containing these four species was tested and found to be effective with 20.0 mm zone of inhibition. This is greater than that of the components. This formulation was found to be effective against *E. coli* (Gram negative) too with value 15.0 mm. [table 2]. The improved value of the formulation must be due to the synergistic effect.

Table 3: Synergistic effect

Sample	*F. ben*	*F. rac*	*F. rel*	*F. mic*	*F. ben+ F. rac*	*F. ben+F. rac+F. rel*	*F. ben+F. rac+F. rel+F. mic*	Ascorbic Acid	*Nalpamaradi . choorna*
Maximum Activity	93.02±0.02 %	83.61±0.03 %	81.00±0.01 %	93.12±0.008 %	87.39±0.04 %	92.33±0.02 %	93.36±0.03 %	93.35±0.04 %	87.30±0.05 %
Conc. µg/ml.	30.00	50.00	50.00	40.00	40.00	20.00	25.00	50.00	50.00
EC_{50}	13.0±0.02	20.0±0.03	16.0±0.04	9.0±0.03	11.0±0.009	6.0±0.03	8.0±0.02	21.0±0.04	29.0±0.03

The synergistic effect can be summarized as follows: *Nalpamaradi. Choorna <(F. ben+F. rac) <(F. ben+F. rac+F. rel+F. mic) <(F. ben+F. rac+F. rel).*

CONCLUSION

The present work highlights the concept of synergy based on the studies of the polyherbal formulations. The formulations studied exhibited synergism of all ingredients with maximum therapeutic efficacy.

Phytochemical analysis of these four species has been reported to show the presence of flavonoids, phenolic acids, tannins, and triterpenoids as major constituents (active principle).

The antioxidant activity is mainly due to the presence of phenolic components, such as phenolic acids, and phenolic diterpenes [31]. The synergistic effect of different combinations has been investigated for the first time. The present investigation has justified and validated the traditional use of these medicinal plants as antibacterial and antioxidant agents. The study has also proved their synergistic effect with improved antioxidant potential in the formulation. Therefore, the traditional Ayurvedic Medicine Nalpamaradi *choorna* may have potential applications in the combination targeted therapies for various neurodegenerative diseases, dermatological treatments, as well as radioprotective medicines, with minimum side effects.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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