International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 1 Jan-Feb 2023, pp: 1609-1616 www.ijprajournal.com ISSN: 2249-7781

"Synergistic Antimicrobial Activity of Bark and Flower of Muntingia Calabura Linn."

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Submitted: 01-02-2023 Accepted: 10-02-2023

ABSTRACT
The objective of the present study is to evaluate the antimicrobial effect of individual as well as combination of bark and flower extracts of Muntingia calabura Linn against few selected pathogens. Both bark and leaf extracts were prepared in aqueous solvent like ethanol in the ratio of 1:20 (w/v) for 72 hours at room temperature. Antimicrobial testing was carried out using agar disk diffusion assay followed by determination of minimum inhibitory concentration (MIC). The microbes targeted were E. coli, B. subtilis, and S. aureus. The test extract exhibited significant ($P < 0.05$) antibacterial effect with the MIC value ranging from 7 to 15µg/ml which indicates that the bark and flower extract could be used as a potential antimicrobial agent and their synergistic effect could be used against antibiotic resistant bacteria.

Keywords: Antimicrobial effect, Muntingia calabura, Agar disk diffusion assay, minimum inhibitory concentration, synergistic effect.

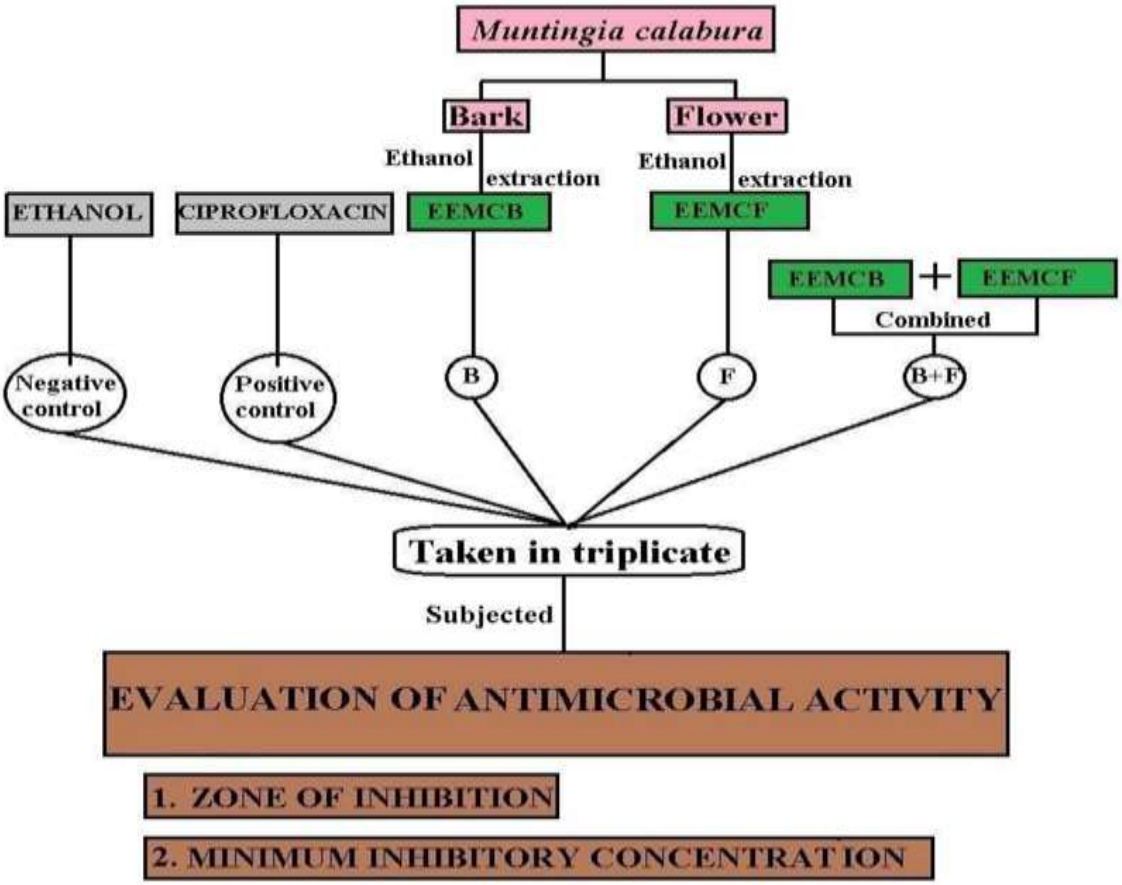

Fig 1: Graphical Abstract

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I. INTRODUCTION

Bacteria evolvedthree billion years ago on the earth; they are exceptionally diverseand present in uncountable numbers. In the past, present and may be in the future some deadly bacterial infections are considered as one of the serious challenge in medical sector. [1]Opportunistic bacteria, such as Pseudomonas aeruginosa, cause illness mostly in immunocompromised individuals. Bacterial infection often causes diarrhoea, pneumonia, infection of skin, urinary tract, and respiratory system especially in children. A person gets bacterial infection when consumed spoiled food, contaminated drinking water or from another infected person. [2]

Antibiotics and antibacterials can be used to treat bacterial infections. Antibiotics can be described as anagent that either stopsgrowth of bacterial (bacteriostatic drugs) or by entirely destroyingthem (bactericidal drugs). [3]

Modern medical procedures depend on the use of antibiotics to control infections, for organ rejection reactions in transplantation and also for surgery. [4]However, infectious illnesses continue to be the leading causes of death in developing nations and the third leading cause of death globally. Also the mortality rate is more compare to that of malaria and AIDS. [5]Irrational use of antimicrobial drugs leads to the development of microbial resistance which made necessary to search for new antimicrobial agents. This resistance phenomenon has become the serious aspect for increased mortality and morbidity rate in both developed and developing country. But the prevalence of bacterial resistance has been increasing even for newly discovered drugs. [6, 7] Bacterial resistance to drugs is an unpredictable threat to the mankind. Regardless of the country, race and climate it affect everyone. [8]

Natural products are those chemical compounds which are available in nature having potential biological or pharmacological activity and are very important target in drug discovery. They are very helpful in the treatment of bacterial infectious without side effects unlike synthetic antimicrobial drugs. Hence, it becomes very necessary to carry out screening of such plants in order to identify the active phytochemical responsible for pharmacological action. [9]In order to find new sources of antibiotics, we must therefore research elsewhere, and naturalsource is a logical starting point.

In India and Malaysia, Muntingia calabura is a popular roadside tree and is commonly grown. In Malay it is called 'Kerukup Siam'. In both Asia and tropical America researchers have reported medicinal uses of various parts of this tree.[10]Since many years, the root of this tree has been used as an emmenogogue and as an abortifacient. Also the flowers of this tree have been used to treat headaches, and as an antidyspeptic, antispasmodic and diaphoretic. Flower infusion is consumed as a tranquillizer and tonic. [11] In addition, the M. calabura leaves extracts also possesses anti-inflammatory, anti-pyretic [12, 13] antibacterial [14] and antistaphyloccocal activity. [15] Therefore, the main objective of this study is to search for the strong synergistic antimicrobial activity which could serve as a good candidate for the development of new antimicrobial agents. This study aimed to evaluate the individual ability of bark and flower extracts as well as their combination against Escherichia coli, Bacillus subtilis and Staphylococcus aureus.

II. MATERIALS AND METHODS

Plant Materials

The flowers and bark of Muntingia calabura were gathered from its natural habitat in K. M. Doddi, Karnataka, India and immediately dried in hot air oven at 120 ^{O}C for 20 minutes. These were previously identified and authenticated from Department of Pharmacognosy, Bharathi College of Pharmacy, under specimen number Bot/2020020.

Preparation of barkextract[16, 17]

Collected bark pieces were immediately washed, air dried and weighed. The dried pieces were ground into powder, sieved (60 mesh) and extracted with ethanol for 48 hours at room temperature. Later, it was filtered by Whatman filter paper no.1 and concentrated under reduced pressure in a rotator evaporator and stored at 4 ^{O}C until further use. The bark extract is coded as MCB.

Preparation of flowerextract[18]

Collected flowers were immediately washed, air dried and weighed. Later they are subjected to drying at 40 ^{O}C for 48 hours. The obtained dried flowers are ground in a mill and weighed again. The flower powder is then transferred to glass beakers and 70% ethanol was added. Maceration is carried out for 72 hours with 24 hours interval for replacing fresh ethanol solvent. The extract was filtered and evaporated. After removal of the solvent, crude ethanol extract

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was obtained which was weighed and stored at 4 ^{O}C until further use. The bark extract is coded as MCF.

Preparation of standard drug

In this trial, a reference antibiotic drug (ciprofloxacin) was employed as well as obtained from the Bharathi College of Pharmacy, Bengaluru, Karnataka. Ciprofloxacin was dissolved in ethanol to obtain the concentration of 0.5 mg/ml. The standard drug, ciprofloxacin is coded as CPRF.

Microorganisms tested

Three human pathogenic microbial strains Escherichia coli, Bacillus subtilis and Staphylococcus aureus were used in the study which were obtained from Microbiology laboratory, department of Pharmcology, Bharathi College of Pharmacy, K. M. Doddi, Maddur.

Antimicrobial screening[19, 20]

Screening for antimicrobial activity of the extracts was done by agar well diffusion method followed by determination of minimum inhibitory concentration (MIC) of the individual as well as combination of bark and flower extracts against the bacterial strains as described by Kirby, 1994. The bacterial strains, Escherichia coli, Bacillus subtilis and Staphylococcus aureus were maintained in nutrient agar slant at 4˚C for use in examining anti-bacterial activity. The prepared flower and bark extracts of their 100 mg/ml concentration were tested for antibacterial activity by the disc diffusion method. The bacterial strains were individually streaked uniformly all over the nutrient agar medium on petri plates. 50 µL of distilled water, standard and test solutions were pipetted onto the wells of 10mm diameter and about 2 cm apart. The plates were incubated at 37 ^{O}C ± 1 ^{O}C for two days to attain good growth. The bacterial zone of inhibition was measured in millimetres (mm) and compared with standard ciprofloxacin. Two petri plates (A & B) were taken for one bacterial species where individual test extract and combination of them were loaded as follows:

Table 1: Petri plate names and contents.

Bacterial species	Petri plate name	Petri plate contents
Escherichia coli	A	MCB, MCF and CPRF
	B	MCB + MCF and CPRF
Bacillus subtilis	A	MCB, MCF and CPRF
	B	MCB + MCF and CPRF
Staphylococcus aureus	A	MCB, MCF and CPRF
	B	MCB + MCF and CPRF

Determination of Minimum Inhibitory Concentration(MIC) for bacteria[21]

In microbiology, minimum inhibitory concentration (MIC) can be defined as the lowest concentration of an antimicrobial (like an antifungal, antibiotic or bacteriostatic) drug that will inhibit the visible growth of a microorganism after overnight incubation. This was calculated from the readings of culture plates after incubation. Each tube is added with respective bacterial species and then incubated overnight. The growth or no-growth was considered by observation, and the MIC value was determined as the lowest extract concentration that avoids the bacterial growth. Distilled water was used as control. Each assay was repeated thrice. After incubation based on turbidity, MIC is calculated.

Statistical analysis

All experiments were carried out in triplicate. Results were presented as mean ± standard deviation (SD). All data were statistically analysed by one-way ANOVA using Graphpad Prism 2019 program to determine whether there were any statistically significant differences between the means of two or more independent groups using P- value ≤ 0.05**.**

III. RESULTS & DISCUSSION

The main objective of the present study was to evaluate the ability of the plants extract to inhibit the growth of pathogenic bacteria and to find out whether the synergistic activity exist between flower and bark extracts. Antimicrobial activity was recorded when the zone of inhibition is greater than 5 mm.

Measurement of antimicrobial activity using Agar well diffusion method

The antimicrobial potential of M. calabura flower and bark as well as their combination was evaluated according to their zone of inhibition

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against the bacterias and the results (zone of inhibition) were compared with the activity of the standard, viz., CPRF.

A. Against Escherichia coli

By disk diffusion method, the effectiveness of MCF, MCB and MCB + MCF were determined against E. coli (Table 3). The combination of flower andbark (MCB + MCF) was exerted highest inhibition zone against E. coli (20 mm) compare to individual but the effect was little lesser than CPRF.

B.AgainstBacillus subtilis

By disk diffusion method, the effectiveness of flower and bark of Muntingia calabura was determined against Bacillus subtilis (Table 3). As shown in Table 3, MCB + MCF was exhibited highest inhibition zone against E. coli (16 mm) compare to individual extract but the effect was much lesser than the CPRF.

C. Against Staphylococcus aureus

By disk diffusion method, the effectiveness of flower and bark of Muntingia calabura was determined against Staphylococcus aureus (Table 3). The MCB + MCF was showed strongest activity than the individual extract but the effect was much lesser than CPRF.

Table 2: Evaluation of zone of inhibition against E. coli, B. subtilis and S. aureus.

Standard/Test	Escherichia coli	Bacillus subtilis	Staphylococcus aureus
	Inhibition zone (mm)		
Control	4.33 mm ± 0.57	9.56 mm ± 0.57	2 mm ± 0.00
Standard	28.66 mm ± 0.57	30.6 mm ± 1.154	28.6 mm ± 0.57
MCF	16.66 mm ± 0.57	7.66 mm ± 0.57	4.33 mm ± 0.57
MCB	14.66 mm ± 0.57	14.33 mm ± 0.57	13.0 mm ± 1.00
MCB + MCF	21.33 mm ±1.52	17.33 mm ± 1.15	18.33 mm ± 0.57

*Data are means of three replicates (n = 3) ± SD. mm= millimeter.

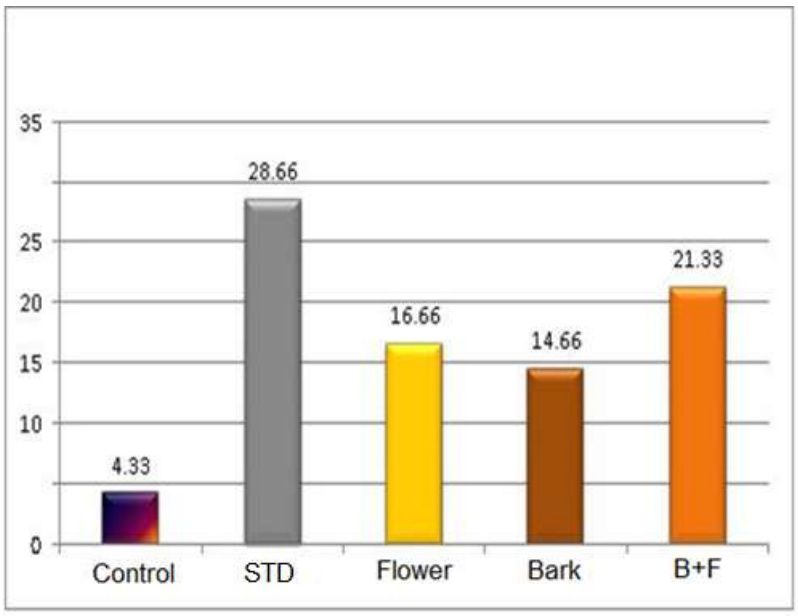

Figure 1: Zone of inhibition against E. coli.

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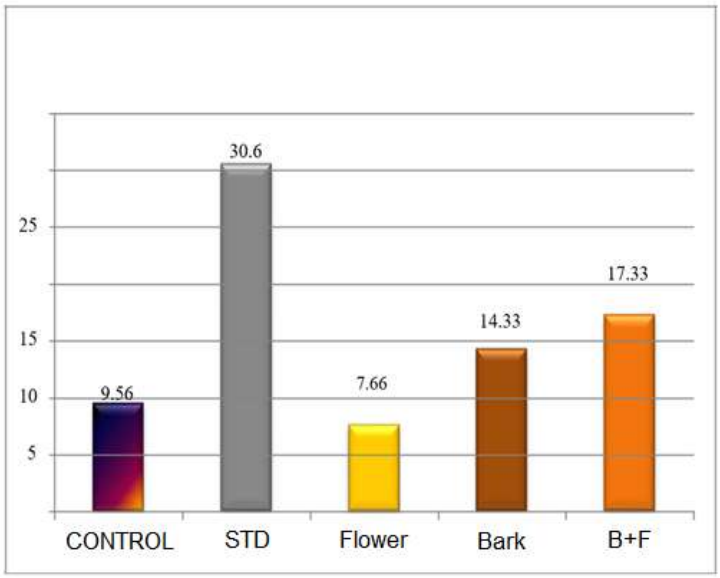

Figure 2: Zone of inhibition against B. subtilis.

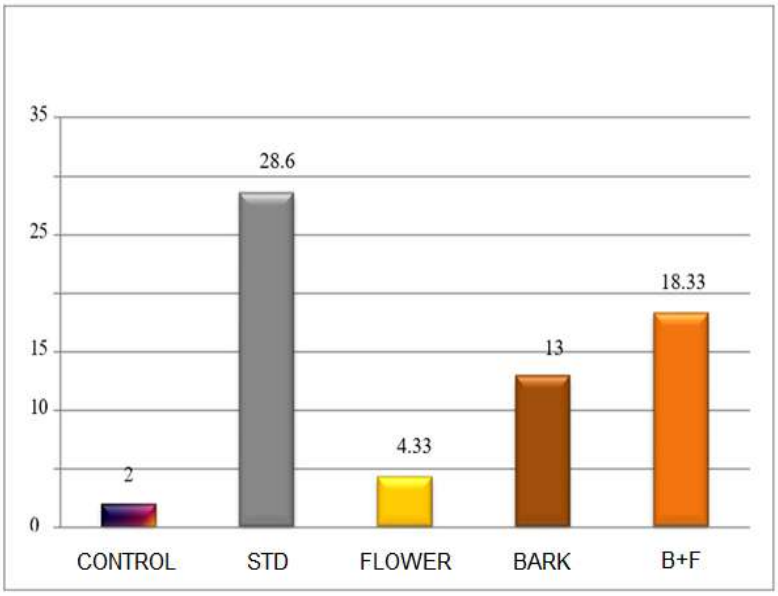

Figure 3: Zone of inhibition against S. aureus.

Determination of minimum inhibitory concentration (MIC)

The results of MIC for different species of bacteria subjected with MCF AND MCB were summarized in Table 3a, b and c. The MCF had shown a high MIC value (15µg/ml) against S. aureus and B.subtilis whereas the MCB alone had shown low MIC value (7.5µg/ml) and the combination of bark and flower had exerted low MIC value which represents high antibacterial effect.

Synergistic activity of bark and flower extract[22, 23]

The MCB + MCF were showed stronger synergistic effect with all three bacterial species compared to their individual effect. The synergistic effect is highest in E. coli (21mm) and lowest in B. subtilis (17mm) in the evaluation of zone of inhibition.

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Table 3a: Minimum inhibitory concentration against E.coli.

Test tube no.	Concentration (µg/ml)	Observation (CPRF)	Observation (MCB)	Observation (MCF)	Observation (B+F)
1	0	Turbid	Turbid	Turbid	Turbid
2	5	Turbid	Turbid	Turbid	Turbid
3	10	Clear	Turbid	Clear	Clear
4	20	Clear	Clear	Clear	Clear
MIC		10+5/2=7.5 µg/ml	20+10/2 =15 µg/ml	20+10/2=15 µg/ml	20+10/2=15 µg/ml

Table 3b: Minimum inhibitory concentration against B. subtilis.

Test tube no.	Concentration (µg/ml)	Observation (CPRF)	Observation (MCB)	Observation (MCF)	Observation (B+F)
1	0	Turbid	Turbid	Turbid	Turbid
2	5	Turbid	Turbid	Turbid	Turbid
3	10	Clear	Clear	Turbid	Clear
4	20	Clear	Clear	Clear	Clear
MIC		10+5/2=7.5 µg/ml	10+5/2=7.5 µg/ml	20+10/2=15 µg/ml	10+5/2=7.5 µg/ml

Table 3c: Minimum inhibitory concentration against S. aureus.

Test tube no.	Concentration (µg/ml)	Observation (CPRF)	Observation (MCB)	Observation (MCF)	Observation (B+F)
1	0	Turbid	Turbid	Turbid	Turbid
2	5	Turbid	Turbid	Turbid	Turbid
3	10	Clear	Clear	Turbid	Clear
4	20	Clear	Clear	Clear	Clear
MIC		10+5/2=7.5 µg/ml	10+5/2=7.5 µg/ml	20+10/2=15 µg/ml	10+5/2=7.5 µg/ml

IV. DISCUSSION

Bacterial infections represent an important cause of illness and deathglobally. As a result, there is growing interest in the development of novel antimicrobial medicines for the treatment of bacterial infections.[24]Phytochemicals derived from natural sources serve as a prototype to produce more effective and less toxic medicines to control the growth of pathogenic microbes. Many studies have been conducted with the various plant extracts toevaluate antimicrobial activity. Therefore, medicinal plants are finding their way into pharmaceuticals, neutralceuticals and food supplements.[25]

In the present investigation, MCF, MCB and MCF+MCB were evaluated for the exploration of their antimicrobial activity against E. coli, B. subtilis, S. aureuswhich were regarded as the human pathogenic microorganism. Susceptibility of each plant extract was tested by agar well diffusion method and determination of minimum inhibitory concentration (MIC).

Our preliminary investigation showed that the combined effect of Muntingia calabura flower and bark (MCF & MCB) was more compare to individual effect against tested bacteria. Hence the plant extracts had synergistic ability to inhibit the growth of microorganism. The antimicrobial analysis using the agar well diffusion method and MIC value is been used by many researchers. [26, 27]In the present study the MIC value of the active plant extracts obtained were lower suggesting that the plant extracts were bacteriostatic at lower concentration.

V. CONCLUSION

The present research was aimed at screening plant extracts and their combination for their antimicrobial activity and antimicrobial potentiating properties, thereby identifying potential plant extracts for further development as safe, effective, affordable, alternative therapeutic agents, most likely new antimicrobials. The objectives have been met to an appreciable extent,

though further research and efforts are warranted to realize the absolute goal.

The plant extracts under investigation showed the presence of several bioactive phytoconstituents and were non-toxic. Among them, the bark and combination of flower and bark extracts showed substantial antibacterial activity against the target bacterial species. The bark extract especially has demonstrated substantial efficacy at the higher tested dose. Hence, the combination of bark and flower has likely potential to be developed as safe and effective antimicrobial therapeutic agent.

It is necessary that research should continue towards isolation and purification of bioactive components from these extracts for use in drug discovery and development in search of newer antimicrobial therapies. The present study has set forth the significance of natural products to control antibiotic resistant bacteria which are being a threat to human health. This scientific study can serve as an important platform for the development of inexpensive, safe, effective and alternative phytomedicines, especially antimicrobials.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Acknowledgement

The authors were thankful to Department of Pharmacology, Bharathi College of Pharmacy, Bharathinagarfor their help extended to us during the entire studies.

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