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Pharmacological investigation of methanol extract of *Syzigum cuminii* seeds and *Crateva nurvula* bark on the basis of antimicrobial, antioxidant and anti-inflammatory properties

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ABSTRACT

Syzygium cumini (syn. Eugenia jambolana) commonly known as "Jamun" having promising therapeutic value with its various phytoconstituents such as Tannins, Alkaloids, Steroids, Flavanoids, Terpenoids, Fatty acids, Phenols, Minerals, Carbohydrates and Vitamins. Crateva nurvula (Varuna) is also known to be used in Ayurvedic medicine since ancient times in India as it also contains different phytochemical constituents. In the present investigation, seeds of Jamun and bark of Varuna were investigated for their antimicrobial, antioxidant and anti-inflammatory potential. Different solvent extracts of both the plant (s) parts were prepared according to decreasing polarity. The methanol extracts of both the plants were found to be most potent antimicrobial, antioxidant and anti-inflammatory agent in comparison to other solvent extracts. Methanol extracts (200 mg/ml) of Jamun showed maximum potency against Aspergillus niger but showed moderate activity against other pathogens. These extracts also showed predominant in vitro antioxidant activity and in vivo anti-inflammatory activity in carrageenan induced animal models at a dose of 120 mg/kg. Methanol extracts (200 mg/ml) of Varuna showed maximum potency against Bacillus subtilis, Salmonella typhii, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans but showed moderate activity against other pathogenic microorganisms. These extracts were having predominant in vitro antioxidant activity and in vivo anti-inflammatory activity at a dose of 50 mg/kg in carrageenan induced animal models. The methanol extracts of the plants were also screened for their hemolytic activity against sheep erythrocytes but the extracts showed negative hemolysis.

Keywords: *Syzigum cuminii* seeds, *Crateva nurvula* bark, antimicrobial activity, antioxidant activity, anti-inflammatory activity.

INTRODUCTION

In developing countries and particularly in India low income people such as farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections [1]. These plants are ingested as decoctions, teas and juice preparations to treat respiratory infection. They are also made into a poultice and applied directly on the infected wounds or burns. Traditional healers claim that some medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases [2]. The country like India is blessed with the natural medicines which are plant derived and these plant medicines are cheap, no side effects and are found to be effective in various resistant pathogenic microorganisms. These are also the good source of antioxidant and also cure various disorders associated with inflammation. Latest and previous studies have concluded the beneficial aspects of plant derived drugs as good source of antibiotics, antioxidants and anti-inflammatory agents [3-7]. Previous studies have reported Jamun and Varuna as natural herbal medicines [8-10].

MATERIALS AND METHODS

All the chemicals and reagents used were from C.D.H and Ranchem. Glass wares used were from Borosil. The media and broth used for microbial culture were from Hi-Media Pvt. Limited, Bombay, India.

Plant material

The authenticated sample was collected from local gardens of Dehradun (U.K), India and was further confirmed in Botanical Survey of India (BSI), Dehradun. Voucher specimens have been deposited in BSI, Dehradun, India.

Preparation of plant extracts

The method [11] was adopted for preparation of plant extracts with little modifications. Briefly four 20 g portions of the powdered plant material were soaked separately in 100 ml of water, hexane, methanol and petroleum ether for 72 h. Each mixture was stirred after every 24 h using a sterile glass rod. At the end of extraction, each extract was passed through Whatmann filter paper no1 (Whatmann, England). The filtrate obtained were concentrated in vacuo using rotary evaporator at 30^{o}C.

Determination of Antibacterial and Antifungal activity

Culture Media

The media used for antibacterial test was soyabean casein digest agar/broth and Sabouraud's dextrose agar/broth of Hi media Pvt. Bombay, India.

Inoculum

The bacteria were inoculated into soyabean casein digest agar/broth and incubated at 37^{0} C for 4 h and the suspension were checked to provide approximately 10^{5} CFU/ml. Similar procedure is done for fungal strains by inoculating in Sabouraud's dextrose broth for 6 h.

Microorganisms used

The test organisms (*Bacillus subtilis* ATCC6051, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* ATCC 23564, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* K-12, *Staphylococcus aureus*, were the bacterial strains obtained from institute of Microbial Technology (IMTECH) Chandigarh, India. The fungal test organisms used for study are *Candida albicans, Aspergillus niger, Saccharomyces cerevisae* and *Penicillium notatum* obtained from pure lab cultures of Roorkee Research & Analytical Laboratories, Roorkee (U.K), India.

Determination of antimicrobial activity

The agar well diffusion method [12] was modified. Soyabean casein digest agar (SCDA) was used for bacterial cultures. The culture medium was inoculated with the microorganism separately suspended in soyabean casein digest broth. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabourauds dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks (distilled water, hexane, methanol and petroleum ether as the case may be). Standard antibiotic (Chloramphenicol, concentration 1mg/ml) was simultaneously used as positive control. The bacterial plates were then incubated at 37^{o}C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The same procedure was done for determining antifungal activity but in this case standard antibiotic (Fucanazole, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37^{o}C for 72 h. Here also the diameter of zone of inhibition observed was measured.

Determination of MIC and MBC

The antibacterial and antifungal plant extracts were then after evaluated to determine MIC and MBC values. The serial dilution technique by using N-saline for diluting the plant extract was adopted and serially diluted plant extract tubes were incubated for 48 h. The minimum dilution of the plant extract that kills the bacterial and fungal growth was taken as MLC (Minimum lethal count) while the minimum dilution of plant extract that inhibits the growth of the organism was taken as MIC.

Determination of cellular toxicity using sheep erythrocytes

The method [13] was employed to study cellular toxicity. Briefly 10 fold serial dilution of the extract were made in phosphate buffered saline. A total volume of 0.8ml for each dilution was placed in an ependroff tube. A negative control tube (containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37^{o}C for 30 minutes and all tubes were centrifuged for 5 minutes and then observed for hemolysis.

Determination of *in vitro* antioxidant activity

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

The extract solution for the DPPH test [14] was prepared by re-dissolving 0.2 g of each dried extract in 10 ml methanol. The concentration of DPPH solution was 0.025 g in 1000 ml of methanol. Two ml of the DPPH solution was mixed with 10, 20 and 40 µl of the plant extract/methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible

Systronics spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

$$\text{Inhibition\%} = (\text{Abst=0 min} - \text{Abst=30 min}) / \text{Abst=0 min} \times 100$$

Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. IC50 is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Determination of Superoxide Anion Radical Scavenging Activity

Superoxide Anion Radical scavenging Activity was measured [15] with some modifications. The various fractions of extracts of the plant (s) were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 µM riboflavin, 0.02 M methionine and 5.1 µM NBT separately. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using Systronics UV-VIS spectrophotometer. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. The reaction mixture without any sample was used as negative control.

The Superoxide anion radical scavenging activity (%) was calculated as:

$$\frac{Ao - As}{Ao} \times 100$$

Where, Ao = absorbance of positive control
As = absorbance of sample

Determination of *in vivo* anti-inflammatory activity

Animals

Male albino rats (180–200 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals [16]. The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1ºC with a 12-h light/dark cycle. The institutional animal ethical committee has approved the protocol of the study.

Carrageenan-induced edema in rats

6 Groups of five animals each were used. Paw swelling was induced by sub-plantar injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. The solvent extracts of *Syzigum cuminii* seeds at dose of 120 mg/kg and *Crateva nurvula* bark at a dose of 50 mg/kg were administered orally 60 minutes before carrageenan injection. Aspirin (10 mg/kg) was used as reference drug. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer at time 0, 1, 2, 3, and 4 h after carrageenan injection.

Statistical analysis

The results were expressed as mean ± S.D. Statistical significance was determined by analysis of variance and subsequently followed by Turkey's tests. P values less than 0.05 were considered as indicative of significance. The analysis was performed using INSTAT statistical software.

RESULTS AND DISCUSSION

Determination of antimicrobial activity

The antimicrobial activity was determined by measuring the diameter of zone of inhibition recorded. Methanol extracts of both the plant (s) parts were found to be most potent antimicrobial agent in comparison to other extracts. Hexane and petroleum ether extracts showed similar antimicrobial activity but less significant in comparison to methanol extracts (results of antimicrobial effect of other solvent extracts are not mentioned). The results are in accordance with the previous studies done on this aspect. The initial screening of antimicrobial activity of extracts were assayed *in vitro* by the agar diffusion method using active against all tested bacterial and fungal strains (**Table 1**). The highest inhibitory effect of methanol extract of *Syzigum cuminii* (Jamun) was observed against *Aspergillus niger* (zone of inhibition: 19.0 mm) while the moderate activity was demonstrated against *Bacillus subtilis, Proteus vulgaris, Salmonella typhii, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Penicillium notatum* (zone of inhibition: 5.0- 9.0 mm). *E.coli* K-12 and *Saccharomyces cerevisae* showed no zones of inhibition against methanolic extracts of Jamun thus were believed to be resistant strains. The highest inhibitory effect of methanol extract of *Crateva nurvula* (Varuna) were found to be against *Bacillus subtilis, Salmonella typhii, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* (zone of inhibition: 10.0- 19.0 mm) but showed moderate activity against *E.coli* K-12, *Proteus vulgaris, Saccharomyces cerevisae, Aspergillus niger* and *Penicillium notatum* (zone of inhibition: 5.0-9.0 mm). In view of the results obtained by the well diffusion method, the minimal inhibitory concentration MIC of methanol extract was determined by broth microdilution assay.

Table 1: Diameter of zone of inhibition (mm) of methanol extract of *Syzigum cuminii* (seeds) and *Crateva nurvula* (bark)

Diameter of zone of inhibition (mm)

Microorganisms	Methanol extract (200 mg/ml)		Control	
	S.cuminii	***C.nurvula***	**C**	**F**
Bacillus subtilis	9.0	16.0	25.8	----
E.coli	NA	9.0	35.0	----
Proteus vulgaris	7.0	8.0	26.0	----
Salmonella typhii	8.0	10.0	27.8	----
Pseudomonas aeruginosa	7.0	17.0	25.0	----
Staphylococcus aureus	5.0	19.0	27.0	----
Candida albicans	7.0	13.0	-----	24.0
Aspergillus niger	19.0	8.0	-----	23.0
Saccharomyces cerevisae	NA	8.0	-----	25.0
Penicillium notatum	6.0	9.0	-----	27.0

C, Chloramphenicol; F, Fucanazole (1mg ml^{-1}); -, Not tested

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

DPPH Radical scavenging activity was determined of each of the fractions of the plants extracts. The experiments were performed in triplicates and mean values of Antioxidant activity of each of the fraction of the plant extract were determined. The four extracts of *Syzigum cuminii* (seeds) and *Crateva nurvula* (bark) tested for antioxidant activity using DPPH radical scavenging method were determined, The four extracts of *Syzigum cuminii* (seeds) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC_{50} values of 33.10 µg/ml and 26.30µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC_{50} values of 43.10 and 36.27 µg/ml. The four extracts of *Crateva nurvula* (bark) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with similar IC_{50} values as that of *Syzigum cuminii* (seeds). The petroleum ether and hexane extracts also showed similar antioxidant activity as that of *Syzigum cuminii* (seeds) with IC_{50} values of 43.10 and 36.27 µg/ml. The known antioxidant ascorbic acid exhibited IC_{50} value of 78.7 µg/ml as shown in **Table 3.**

Table 3: Antioxidant Activity by DPPH Radical Scavenging Method

Plant (s)	DPPH-Radical Scavenging Method (IC50); µg/ ml			
	M	W	P	H
Syzigum cuminii	33.10	26.30	43.10	36.27
Crateva nurvula	-do-	-do-	-do-	-do-

Ascorbic acid = 78.17 µg/ml, The results are the average of three determinations for extracts.

Determination of Antioxidant activity by Superoxide Anion Radical Scavenging Activity

Superoxide anion radical scavenging was determined of each of the fractions of the plant extracts. The experiments were performed in triplicates and mean values of antioxidant activity of each of the fraction of the plant extract were determined. The values of antioxidant activity determination by Superoxide Anion Radical Scavenging method follow the same order as that of DPPH assay. Among the four extracts of *Syzigum cuminii* (seeds) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 67.88 % and 65.34 % inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 58.87% and 55.08% inhibition. Among the four extracts of *Crateva nurvula* (bark) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 72.3% and 68.67% inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 73.5% and 72.14% inhibition. The known antioxidant ascorbic acid exhibited 87.8% inhibition as shown in **Table 4.**

Table 4: Antioxidant Activity by Superoxide Anion Radical Scavenging Method

Plant (s)	Superoxide Anion Radical Scavenging Activity (%)			
	M	W	P	H
Syzigum cuminii	67.88	65.34	58.87	55.08
Crateva nurvula	72.3	68.67	73.5	72.14

Ascorbic acid = 87.80%, The results are the average of three determinations for each of the extracts

Determination of Anti-inflammatory activity

Carrageenan-induced edema in rats

The anti-inflammatory effects of the solvent extracts of both the plant(s) extracts on carrageenan-induced edema in rat's hind paws are presented in **Table 5 and 6**. The anti-inflammatory activities of both the plant (s) parts extracts were found to have effect in dose-dependent manner. There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, *Syzigum cuminii* (seeds) methanol extract and aqueous fractions (120 mg/kg) showed a significant reduction in the edema paw volume. In the test groups treated with *Crateva nurvula* (bark) methanol extract and aqueous fractions (50 mg/kg) showed reduction in edema paw volume. There was no reduction in inflammation found in case of rats treated with petroleum ether and hexane extracts of both the plants. The values of reduction in paw volume, 0.10 ± 0.002, 0.14 ± 0.002 and 0.16 ± 0.002 were found significantly of methanol extract, aqueous extract of seeds of *Syzigum cuminii* and aspirin, respectively at 4 h after carrageenan administration. The values of reduction in paw volume, 0.08 ± 0.002, 0.10 ± 0.002 and 0.16 ± 0.002 were found significantly of methanol extract, aqueous extract of bark of *Crateva nurvula* and aspirin, respectively at 4 h after carrageenan administration. The present study provides evidence that the methanol fraction and aqueous extract of seeds of *Syzigum cuminii* and bark of *Crateva nurvula* acts as potent anti-inflammatory agent in rats in acute inflammation model.

Table 5: Anti-inflammatory activities of different extracts of seeds of *Syzigum cuminii*
Paw volume (ml) ± SD

Experiment	Control	Aspirin (25mg/kg orally)	Methanol extract (120 mg/kg)	Aqueous extract (120mg/kg)	Petroleum ether (120mg/kg)	Hexane (120mg/kg)
1h after treatment	0.25±0.003	0.21±0.003	0.23± 0.003	0.28±0.003	0.20±0.003	0.34±0.003
2h after treatment	0.25±0.003	0.18±0.003	0.20± 0.003	0.24±0.003	0.15±0.003	0.34±0.003
4h after treatment	0.25±0.003	0.16±0.002	0.10± 0.002	0.14±0.002	0.30±0.002	0.34±0.002

±, S.D, Standard Deviation

Table 6: Anti-inflammatory activities of different extracts of bark of *Crateva nurvula*
Paw volume (ml) ± SD

Experiment	Control	Aspirin (25mg/kg orally)	Methanol extract (50 mg/kg)	Aqueous extract (50mg/kg)	Petroleum ether (50mg/kg)	Hexane (50 mg/kg)
1h after treatment	0.25±0.003	0.21±0.003	0.23± 0.003	0.28±0.003	0.20±0.003	0.34±0.003
2h after treatment	0.25±0.003	0.18±0.003	0.20± 0.003	0.24±0.003	0.18±0.003	0.32±0.003
4h after treatment	0.25±0.003	0.16±0.002	0.08± 0.002	0.10±0.002	0.20±0.002	0.34±0.002

±, S.D, Standard Deviation

CONCLUSION

The extracts of the plant (s) part used showed prominent antioxidant and anti-inflammatory activity. The use of these plants in the treatment of pathogenic diseases associated with the infection of these pathogens, as well as an antioxidant and potent anti-inflammatory agent is validated, scientifically supported by the results obtained in this work.

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