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Assessment of *In vitro* Antimicrobial and Antioxidant Potential of *Thevetia neriifolia,* Juss. Flower Morphoforms

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Abstract : *On the basis of flower colour, the Apocynacean member Thevetia neriifolia,Juss. can be grouped into three morphoforms, viz. yellow, orange and white. Preliminary studies of different fractions of these three flower extracts (Petroleum ether (PE), Chloroform (CH), Ethyl Acetate (EA) and Methanol (MT) were carried out using Agar well diffusion method to evaluate the in vitro efficacy of antimicrobial properties against different pathogenic strains (Staphylococcus aureus, Streptococcus pyogenes, Nocardia asteroids, Pseudomonas aeruginosa, Proteus mirabilis, Candida albicans and Trichophyton rubrum) that cause human skin infections. Assays were carried out using different concentrations (10-1.25mg/50µl/6mm well) of the fractions and all organisms responded well in a dose dependent manner. Among the fractions, EA fraction showed maximum activity against N. asteroids (24.00±0.00 - 25.33±0.58mm) followed by P. mirabilis (21.33±1.15 - 22.33±1.15mm) and P. aeruginosa (19.67±1.53 - 21.00±1.41mm). MT fraction responded much better than CH fraction. PE fraction showed least activity against most of the tested pathogens. Assessment of antioxidant potential was done using DPPH free radical assay. EA fraction recorded a higher percentage of antioxidants with IC_{50} values 0.11-0.32mg/ml, followed by MT (0.3 - 0.45mg/ml) and CH (0.47 -0.9 mg/ml) fractions. Results of the present investigation revealed that flower extracts can be used as a good source of antimicrobial agent and as a natural antioxidant, justified the folkloric use of this medicinal plant.*

Keywords: *antimicrobial, antioxidant, flower morphoforms, skin pathogens, Thevetia neriifolia*

I. INTRODUCTION

The tribal people and ethnic races throughout the world use medicinal plants as remedies against many infectious diseases since ancient time. Many plants are used for a wide variety of pharmaceutical purposes as they contain a broad array of biomolecules. Most of the plants in nature have curative properties in one way or the other. The curative efficacy often lies in the secondary metabolites that are distributed to various tissues or organs. *Thevetia neriifolia,* Juss. (Family Apocynaceae), one of the key medicinal plants grown worldwide is reported to have various therapeutic properties in Traditional system of medicine in many countries, including India and China.

Thevetia neriifolia (syn. *T. peruviana*) is a perennial evergreen shrub, grows up to a height of 20-22 feet and possesses large attractive funnel shaped flowers in various colors. The plant is well known for its toxic properties rather than its therapeutic value because it is regarded as a store house of various biologically active secondary metabolites like cardiac glycosides, thevetin A and B, thevetoxin, peruvoside, iridoid glycosides, cerberin, quercetin, flavonoids, triterpenes, monoterpenes, thevefolin, ruvoside, nerifolin and oleandrin [1]. Whole plant and various plant parts have been used in various formulations as a purgative, diuretic, cathartic and febrifuge to treat bladder stones, edema, intermitted fevers, insomnia, hemorrhoids, tumors, rheumatism, dropsy, ulcers, leprosy, ringworm, etc[2-5]. Pragati *et al* [6] revealed the presence of alkaloids, glycosides, phenolic compounds, tannins, oils and gums in methanol extracts of fresh *Thevetia* flowers. Different active compounds were used as insecticides, fungicides, rodenticides and bactericides [7-10], clearly indicate the presence of diverse secondary metabolites in various plant parts.

The plant thrives well in moist sandy soil and is cultivated as an ornamental or hedge that blooms throughout the year. Flowers are generally bright yellow, but orange and white forms are also occasionally cultivated. Yellow forms are sweet scented, while the other two variants lack fragrance. Although there have been many reports on the pharmacological benefits of various plant parts of *Thevetia* sp. elsewhere, information regarding the antioxidant and antibacterial activities of these three flower morphoforms is very limited. Present study was therefore undertaken to assess antimicrobial activity and antioxidant potential and to evaluate the better efficacy within the three morphoforms.

II. Materials & Methods

Withered flowers were collected from different localities of Trichur Dist, Kerala, from their natural habitat, immediately after dropping out of the plant. The plant specimens were authenticated by Dr. Sunil C N, Associate Professor in Botany, S N M College, Maliankara, Ernakulam and the voucher specimens were deposited in the Herbarium of Botany Department, St. Teresa's College, Ernakulam.

The samples were washed thoroughly and dried in shade for about two weeks. Accurately weighed powdered samples (40g) were subjected to successive soxhlet extraction using 350 ml each of petroleum ether (PE), chloroform (CH), ethyl acetate (EA) and methanol (MT) in a soxhlet apparatus. Cold extraction was also carried out using 5 g of dried powder with 50 ml of 70% Methanol using a magnetic stirrer for 24 hours. Extracts were concentrated and dried fractions were stored in labeled air tight containers at 4^oC for further studies.

Microorganisms for the present study were collected from MTCC, Chandigarh and from the Department of Microbiology, Amala Medical Institute of Sciences, Trichur. Three gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, and Nocardia asteroids*) and two gram negative bacteria (P*seudomonas aeruginosa* and *Proteus mirabilis*) were selected for antibacterial studies. Antifungal activities were evaluated using *Candida albicans* and *Trichophyton rubrum*.

Assays for antimicrobial evaluation were conducted by the standard protocol of Agar well diffusion method [11]. Nutrient Agar medium was prepared as per the recommendations of the manufacturer (Hi media Laboratories Pvt. Ltd. Mumbai, India). A stock solution of 200mg/ml of all the extracts were prepared and diluted serially to get varying concentrations of 100, 50 and 25mg/ml. Inoculated petri-dishes were loaded with fractions of 10-1.25mg/50 µl/6 mm wells. After the allotted incubation time at 37^oC, measured the zone of inhibition in millimeter. Dimethyl sulfoxide (DMSO), Gentamicin and Fluconazole (25-50µg/well) were used as negative and positive controls for bacteria and fungi respectively. Results were analyzed by comparing the efficacy of extracts with standard antibiotics. Bioassays of all four fractions (PE, CH, EA & MT) of yellow form was evaluated initially and compared with that of orange and white flower variants to assess better activity.

Free radical scavenging assay of each fraction was carried out by the DPPH method of Molyneux [12]. The reaction volume contained 0.9ml of 1.5mM DPPH in methanol and 0.1ml of extracts of different concentrations (0.1-1.2mg/ml). Absorbance was determined spectrophotometrically at 517 nm after 20 min. of incubation time and IC_{50} values were calculated using the formula (Ab. of blank – Ab. of sample)/Ab. of blank x 100. Both the assays were conducted in triplicate and the mean values and standard deviation was analyzed statistically.

III. RESULTS AND DISCUSSIONS

With all four fractions, antimicrobial and antioxidant potential of flowers from three morphovariants were analyzed by well diffusion method against seven different pathogenic strains that causes human skin infections. All microbes responded very poorly at low concentrations (1.25-2.5mg/well), but showed moderate activity at medium concentration (5mg/well). However, at a concentration of 10mg/well all organisms showed better sensitivity towards all fractions except PE. Hence, only higher concentration was studied for evaluating better response among color variants (Fig 1 A-H).

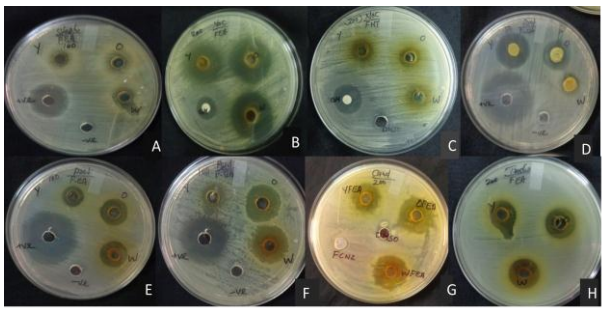
The results clearly indicated that most of the tested organisms showed maximum resistance against PE fraction (Table 1) of yellow forms, and the other two forms also exhibited almost the same result with minimum inhibition zones. Amongst CH fractions, the most remarkable sensitivity was expressed by *P. mirabilis* of yellow array preceded by orange and white forms. Gram positive bacteria *S. aureus* has shown a significant inhibition zone with this fraction as compared to standard Gentamicin.

TABLE 1 Antimicrobial Activity of PE & CH fractions using Agar Well Diffusion Method (10mg /50µl/well and Zone of inhibition in mm)

Organisms	PE(Y)	CH(Y)	CH(O)	CH(W)	+ve
Gram positive Bacteria					Gentamicin
S. aureus	0.00±0.00	12.67 ± 0.58	13.00±1.00	12.33±0.58	14.33±2.08
S. pyogenes	0.00±0.00	13.00 ±0.00	11.33± 0.58	11.67±0.58	13.33±0.58
N. asteroids	9.00 ± 0.00	12.00 ± 0.00	11.00± 1.00	11.00± 0.00	21.67±0.58
Gram negative Bacteria					
P. aeruginosa	10.00 ±1.73	13.67 ± 0.58	12.67± 0.58	11.67± 0.58	25.33±0.58
P. mirabilis	0.00±0.00	21.00 ± 1.00	18.33 ±0.58	14.17± 0.76	28.33±0.58
Fungal strains					Fluconazole
C. albicans	10.00± 0.00	11.17 ± 0.29	11.67± 0.58	12.50± 0.50	12.33±0.58
T. rubrum	0.00±0.00	12.17 ± 0.29	11.17± 0.76	10.33± 0.29	13.33±0.58

PE(Y) - Petroleum Ether fraction of Yellow form, CH (Y, O, W) - Chloroform fractions of Yellow, Orange & White forms.

According to the results presented in Table-2, EA fraction was the most effective solvent in extracting an array of antimicrobials, whereas multi resistant strain *S. aureus*, a common causative agent for many diseases was least sensitive. Except this strain, all the six organisms studied were shown remarkable sensitivity to this fraction with excellent inhibition zones ranging from 15 – 25 mm. All the microbes responded intermediately towards the MT fraction. The type of solvent plays a major role in extracting various secondary metabolites with antimicrobial properties and this accounts for the variations noted in the inhibition zones by the same organism against different fractions.



A- *S. aureus*(EA-5mg) B- *N. asteroids* (EA-10mg) C-*N.asteroids* (MT-10mg) D- *P. mirabilis* (CH-2.5mg)
E- *P.* mirabilis (EA-5mg) F- *P. aeruginosa*(EA-10mg) G –*C. albicans* (EA-10mg) H- *T. rubrum*(EA-10mg)

Fig -1(A-H) Comparative Evaluation of Antimicrobial Activity of *T. neriifolia* Juss. Flower Extracts Against Human Skin Pathogens

Bhuvaneswary *et al*, [13] identified different classes of bioactive compounds in leaf extracts of this plant. Alkaloids, phenolic compounds and flavonoids were reported in *Thevetia* flower methanol extract by Britto & Gracelin [14]. Results of the present study revealed that the flower extracts are capable of curing many of the human skin infectious diseases caused by harmful pathogenic microbes, as do other parts of the plant [15-17]. Flower extract of *Catharanthus roseus*, (another member of Apocynaceae family) is reported to possess wound healing potential in Sprague Dowley rats [18]. Flowers are rich in flavonoids and these polyphenols present in the edible flower of *Sesbania grandiflora*, were reported to inhibit significantly the growth of some common pathogens including *S. aureus*. Polyphenolics like quercetin, kaempferol and quercetin 7-o-galactoside in the fresh flowers of *Thevetia peruviana* was isolated by Thilagavathi *et al*, [19].

TABLE 2 Antimicrobial activities of EA & MT fractions using Agar Well Diffusion Method (10mg /50µl/well and Zone of inhibition in mm)

Organisms	EA			MT			+ve control
	Y	O	W	Y	O	W	
Gram positive bacteria							Gentamicin
S. aureus	10.83±0.29	10.33±0.58	9.33±0.58	12.33±0.58	9.33±0.58	10.00±1.73	14.33±2.08
S. pyogenes	15.00±0.00	16.33±0.58	17.67±0.58	12.00±0.00	13.33±1.15	12.50±0.71	13.33±0.58
N. asteroids	24.00±0.00	24.67±1.15	25.33±0.58	12.33±0.58	11.67±0.58	10.33±0.58	21.67±0.58
Gram negative bacteria							
P. aeruginosa	19.67±1.53	20.33±1.53	21.00±1.41	11.33±1.53	15.00±1.00	15.33±1.15	25.33±0.58
P. mirabilis	17.67±1.53	21.33±1.15	22.33±1.15	13.67±0.58	12.00±1.00	11.67±0.58	28.33±0.58
Fungal strains							Fluconazole
C. albicans	17.67±0.58	21.00±1.00	19.00±1.00	13.00±1.73	12.67±1.53	11.33±0.58	12.33±0.58
T. rubrum	17.17±1.26	17.33±0.58	17.33±0.58	11.67±0.58	12.00±0.00	12.00±1.00	13.33±0.58

EA – Ethyl Acetate, MT- Methanol fractions of Yellow (Y), Orange (O) and White (W) forms.

These polyphenols are highly beneficial for the prevention of diseases associated with oxidative stress such as cancer, cardiovascular and neurodegenerative diseases [20]. Increased antimicrobial agents along with antioxidants in the extract may act together for the healing property of skin or wound infections caused by these pathogens. Antioxidant assay of yellow flowers (Fig 2) revealed that the EA fraction exhibited high inhibition activity than MT, CH and PE fractions with an IC_{50} value of 0.32 mg/ml. But the crude sample of methanol cold extraction was very poor in antioxidants as it required nearly1 mg to neutralize half of DPPH molecules clearly

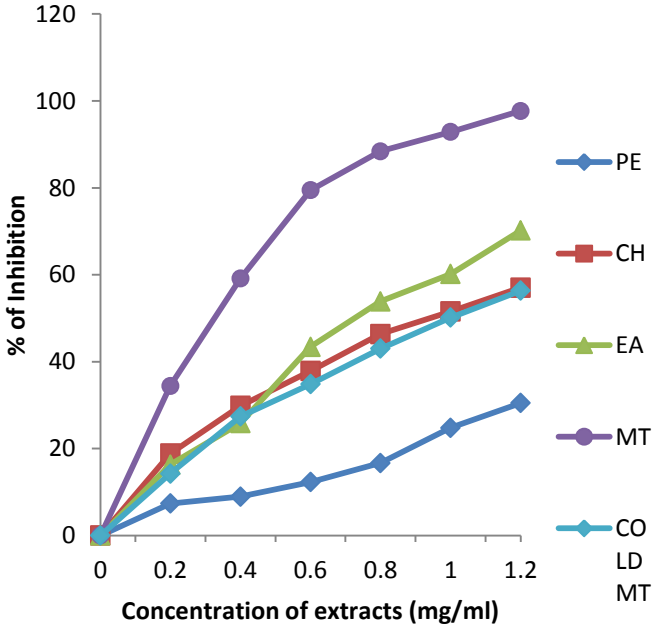


Figure 2. Antioxidant Assay of yellow flower extracts with IC_{50} values (MT - 0.32mg/ml, EA - 0.72mg/ml, CH - 0.9mg/ml, PE >1mg/ml & cold MT 0.99mg/ml)

indicated that hot solvents can isolate various active compounds from the common pool. Among the four solvents used, better activity was expressed by the EA fraction with good IC_{50} values (Fig 3), followed by MT (Fig 4) for all three studied morphoforms. Within the EA fractions, white variant showed excellent antiradical potency followed by orange and yellow forms. The PE fraction of all forms expressed poor activity. Even though the values are well above the standard ascorbic acid (2.5µg/ml), the isolation and purification of the bioactive secondary metabolites add more backing to the therapeutic field. Several antioxidants have been found to be pharmacologically active as prophylactic and therapeutic agents for several diseases. Of course, they are intimately involved in the prevention of cellular damage - the common pathway for cancer, aging, and a variety of diseases [21].

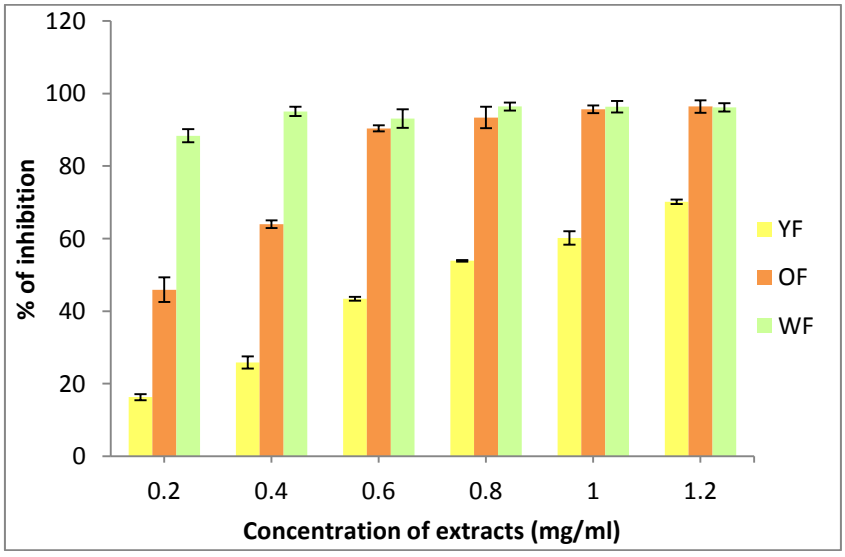


Figure 3. Antioxidant Assay of EA fraction of yellow, orange, and white forms with IC_{50} values (YF- 0.72mg/ml, OF- 0.23mg/ml, WF-0.11mg/ml)

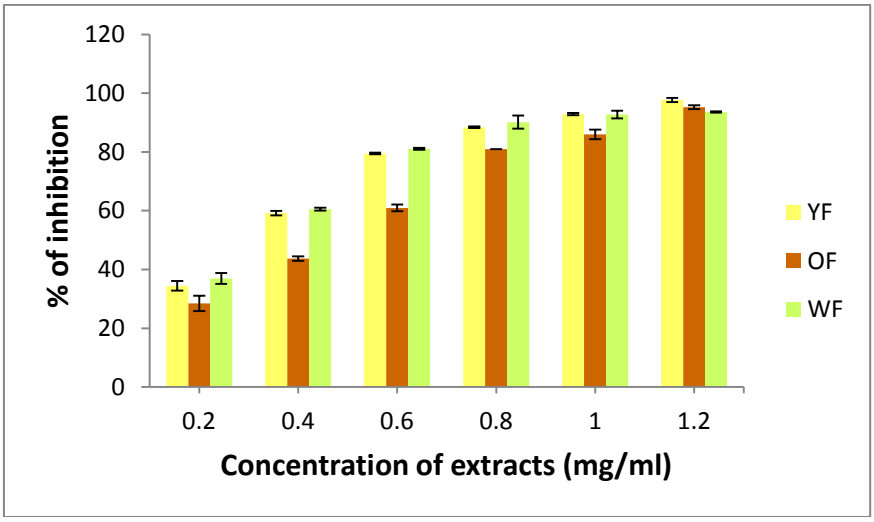


Figure 4. DPPH Assay of MT fraction of yellow, orange, and white forms with IC_{50} values (YF - 0.32 mg/ml, OF- 0.45 mg/ml, WF-0.30 mg/ml)

IV. CONCLUSION

The study revealed that the flower fractions especially EA and MT are very good sources of antimicrobials and natural antioxidants. Among the three forms, white flower seems to be more potent than the other two when both assays conducted. The results support the folkloric use of these plants in the treatment of several infections related to skin diseases and free radical mechanisms. So, the flower extract can be used as a biologically safer and cheaper alternative to the high cost pharmaceutical remedies. The results justified the traditional use of plant extract in the treatment of skin diseases that would protect the skin against bacterial and fungal infections. From these results it is recommended strongly that various fractions of *Thevetia* flower morphoforms can be used in herbal skincare preparations in the form of lotions, soaps, gels, creams or any other external applications to cure skin diseases.

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