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Research Article

A COMPARATIVE EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *THEVETIA NERIIFOLIA*, JUSS FRUIT RIND EXTRACTS

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ABSTRACT

Antimicrobial potential and antioxidant activities of three morphoforms of *Thevetia neriifolia,*Juss fruit wall extracts were evaluated using Agar well diffusion method and DPPH free radical scavenging assay. *In vitro* antimicrobial activities of successive hot extracts (petroleum ether, chloroform, ethyl acetate and methanol) were carried out against seven pathogenic microbes that cause major human skin infections such as cellulitis, impetigo, abscesses, ringworm and folliculitis. Tested microbes include gram positive and gram negative bacterial and fungal cultures: *Staphylococcus aureus, Streptococcus pyogenes*, *Nocardia asteroids, Pseudomonas aeruginosa*, *Proteus mirabilis, Candida albicans* and *Trichophyton rubrum*. Different concentrations of the extract showed considerable activity against most of the microbes in a doze dependant manner. Ethyl acetate fraction recorded a remarkable sensitivity of 13-22mm inhibition zone against the entire tested organisms except *S. aureus* at a concentration of 10mg/50µl/6mm well. Methanol and chloroform fractions established moderate sensitivity, where as petroleum ether fraction was almost ineffective against most of the pathogens. However, DPPH free radical scavenging activity exhibited 50% inhibition for ethyl acetate fraction at a concentration of 1.2mg/ml. Even though the results reveal a moderate amount of antimicrobials and antioxidants in fruit rind, its natural availability add more value to the therapeutic field against skin infections.

Keywords: *Thevetia neriifolia*, Fruit rind, Morpho-variants, Antimicrobial, Human skin pathogens, DPPH assay.

INTRODUCTION

Thevetia neriifolia, Juss (syn *T.peruviana*) commonly known as 'yellow oleander' or 'lucky nut' belonging to family Apocynaceae is a small ornamental tree, said to be a native of Mexico and Central America, is now naturalized everywhere throughout the tropical and subtropical regions. Similar to other members of the family (*Vinca rosea* and *Rauvolfia serpentine*), this plant also contains powerful drugs (Abe *et al*, 1996) with various pharmacological activities. Tribes use medicinal plants in the form of decoction, powder, paste, tinctures and extracts. Folk medicine has used the sap of *Thevetia* to treat aching teeth, chronic sores, ulcers and mange. The bark, leaves, roots and seeds, although often as toxic, has been used in various formulations to treat bladder stones, edema, fevers, insomnia, hemorrhoids, malaria, snake bites, leprosy, scorpion stings, ringworm and other skin diseases (Nellis 1997, Udayan & Balachandran 2009). Plant based natural constituents can be derived from any part of the plant like bark, leaves, roots, fruits, seed, fruit rind, etc (Gordon & David 2001). Antimicrobial properties of *Thevetia* leaf (Bhuvaneswari *et al,* 2011, Mathuravalli & Lakshmi 2012) and seed kernel extracts (Sharma *et al*, 2012, Ravikumar patil *et al,* 2007, Hammuel *et al*, 2011) have proved significant activity by many workers. Flesh of the fruit is presumed to be edible by birds and livestock, but the reports are imprudent as the plant is well known for its toxic properties than therapeutic values (Nellis, 1997).

Yellow oleander, a perennial evergreen shrub cultivated for medicinal purposes, comprises narrow lanceolate leaves and large attractive flowers in terminal cymes. The flowers are generally bright yellow, but orange and white forms also occasionally cultivated. These three flower forms are considered as morpho-variants. Plants bloom throughout the year; fruits are fleshy drupes, thick, succulent, angled, 5-6 cm in diameter, broadly obovate to rhomboidal in shape with a fissure on the ventral side where it dehisces. The immature greenish white fruits become brownish black on ripening. It has a persistent calyx and 3-4cm long peduncle. Fruit wall (rind) consists of 3 layers – upper light greenish waxy pericarp or epicarp, middle fleshy mesocarp and inner hard and stony endocarp. On decorticating the pulpy meso-pericarp (hull), endocarp is exposed which is referred to as nut. Each fruit contains a nut which is longitudinally and transversely divided. After turning black and falling to the ground, the fleshy rind rots away within a week leaving behind a dry nut containing two-four seeds.

All parts of this plant are reported to possess antibacterial, antifungal, cytotoxic, anti-inflammatory, insecticidal and anti HIV properties (Bai & Koshy 2004, Kareru *et al,* 2010). Among bacterial pathogens, *Staphylococcus aureus* and *Streptococcus* can cause a wide variety of infectious manifestations including impetigo, skin abscesses called furuncles and carbuncles, erisepelas, cellulitis and lymphangitis (Stulberg *et al*, 2002). Major fungal infections include candidiasis and ring worm caused by *Candida albicans* and *Trichophyton rubrum.* In spite of the therapeutic and toxicological claim of *Thevetia* fruit especially the seeds, there are no reports in literature regarding the bioactivity of the fruit wall. Hence the present study was undertaken to reveal antibacterial and antifungal properties of various extracts against selected pathogens that cause skin infections and to analyze the antioxidant potential of rind extracts using commercially available DPPH.

MATERIALS AND METHODS

Fresh fruits of *Thevetia neriifolia* were collected from different localities of Trichur dist, Kerala. The plants were authenticated and the voucher specimens were kept in the Herbarium, Department of Botany, St. Teresa's College, Ernakulam. Mature fruits were either plucked from the plant or fallen fruits were collected from the ground, washed thoroughly and kept in shade for 2-3 days for easy separation of fleshy mesocarp through the ventral suture from the stony endocarp. These pulpy fruit rind were dried in shade for 3-4 weeks. Accurately weighed powdered sample (40g) were subjected to successive soxhlet extraction using 350 ml of Petroleum Ether (60-80^{0}C), Chloroform (CH), Ethyl Acetate (EA) and Methanol (MT) for 15-18 hours (Harborne, 1973). All solvents used were analytical grade (MERCK). The fractions were dried, weighed and kept at 4^{0}C for further analyses.

Pure cultures of all experimental bacteria and fungi were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh and from Amala Medical Institute of Sciences, Trichur. Three gram positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Nocardia asteroids*) and two gram negative bacteria (P*seudomonas aeruginosa*, *Proteus mirabilis*) were selected for antibacterial studies. Antifungal activities were evaluated using *Candida albicans* and *Trichophyton rubrum*. All the selected strains are associated with skin diseases.

In vitro antimicrobial assay of all four fractions of three forms were carried out by agar well diffusion method (Navarro *et al*, 2004). As per the recommendations of the manufacturer (Hi media), nutrient agar medium was prepared and poured into UV sterilized disposable petridishes (Tarson). The inoculated plates were loaded with samples of varying concentrations from 1.25-10mg/50µl in DMSO into 6mm wells. After suitable incubation period at 37^{0}C, zone of inhibition was recorded in millimeters. Gentamicin, Fluconazole (25- 50µg/well) and DMSO (Dimethyl sulphoxide) were used as positive and negative controls respectively.

Free radical scavenging activity of different concentrations of all four fractions (0.2-1.2 mg/ml) of three morphoforms were measured against DPPH (2,2 diphenyl-1-picryl hydrazyl-Sigma)) at 517nm spectrophotometrically (ELICO), after 20 min of incubation period (Aquino *et al,* 2001). Percentage of inhibition was recorded according to the standard procedure and IC_{50} value was calculated.

Statistical analysis: All data are expressed as mean ± Standard deviation.

RESULTS AND DISCUSSION

In vitro assays were conducted using all four fractions of rind extracts from three morphovariants. Extraction using petroleum ether (PE) resulted a semisolid sticky paste (1-3%), while CH yielded a thick solid residue (2-2.5%). Similarly, an oily brownish paste was obtained for EA fraction (5-9%) and a semisolid brown mass with a maximum yield of 45-60% was collected using polar solvent MT implying that maximum active compounds can be extracted using polar solvents.

With all four fractions, the antimicrobial activity of fruit rind from three morphovariant plants were analyzed by well diffusion method against seven different pathogenic strains that causes human skin infections. A mother solution of 200mg/ml was prepared and serial dilutions were made to get varying concentration of 100-25mg/ml. These four different concentrations ranging from 10 – 1.25mg/50µl/well were tested for yellow flowered form against all organisms and negligible susceptibility (Fig1 a-b) was noticed at low concentration (1.25 & 2.5mg/well), moderate in medium concentration (5mg/well), but excellent inhibition was recorded against most of the organisms (Fig 1c-f) at higher concentration (10mg/well). So, only the highest concentration was tested for orange and white forms.

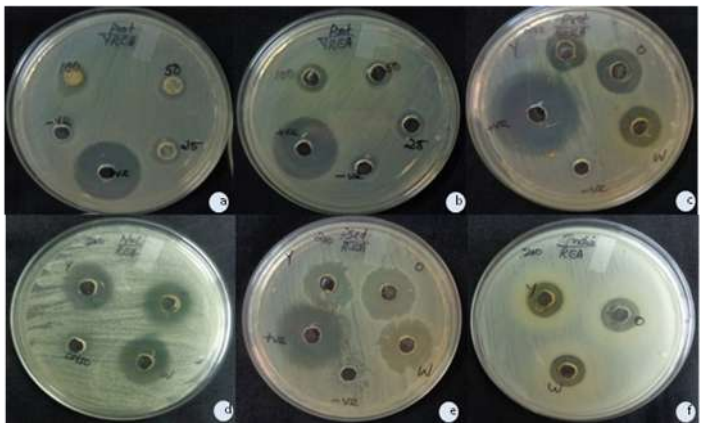

Fig. 1: a-f Antimicrobial activity of Thevetia neriifolia fruit rind fractions of three morphoforms by well diffusion method

a -b) *Proteus mirabilis* (YRCH & YREA - 1.25, 2.5 & 5mg/well, c) *P. mirabilis* d) *Nocardia asteroids*, e) *Pseudomonas aeruginosa* f) *Trichophyton rubrum* (EA fractions at 10mg/well of Yellow(Y), Orange (O) and White (W) forms.

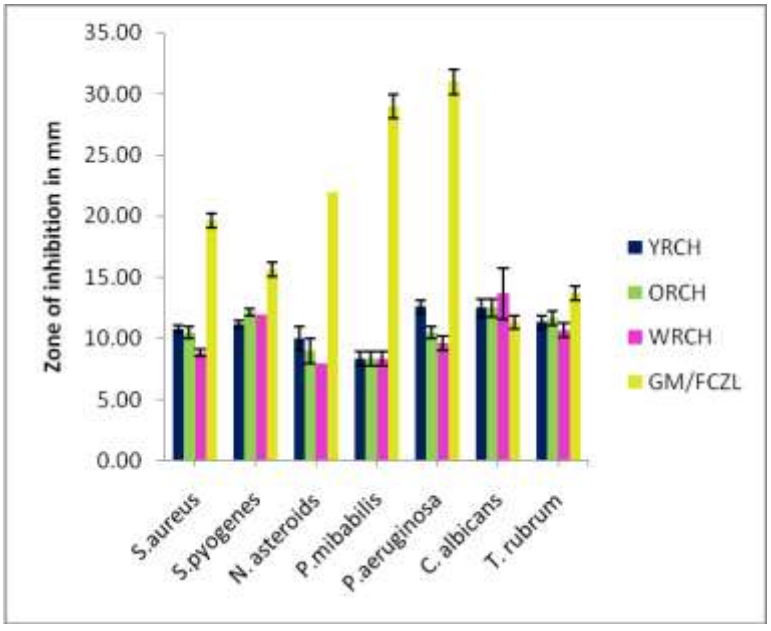

Fig. 2: Graphs showing antimicrobial activity of chloroform fraction of *T. neriifolia* rind extracts on different human skin pathogens

Petroleum ether fraction could not inhibit the growth of any strain with the exception of *P. aeruginosa.* The results presented in Fig 2 explain the inhibition zones of microbes caused by CH fraction of all forms along with standards. Gram positive bacteria *S. pyogenes* was highly sensitive to CH fraction of all forms while comparing the inhibition zone produced by antibiotic Gentamicin. Likewise, both the fungal cultures - *C. albicans* and *T.rubrum* responded moderately to CH fraction. MT fraction was equally effective as CH fractions, but EA fraction proved their ability to control the growth of pathogenic microbes more efficiently with inhibition zones ranging from 13-22mm (Table 1). Among the gram positive bacteria, highest sensitivity was shown by *N. asteroids* (Fig 1d) followed by gram negative *P. aeruginosa* (Fig 1e) and *P. mirabilis* (Fig 1c). Comparing the sensitivity of microbes with MT fraction revealed that this doze responded effectively against all pathogens with a zone of inhibition of 9-14mm (Table 2). The methanol extract of pomegranate rind was proved to be very effective against a wide range of microbes including *S. aerues* (Dahham *et al*, 2010). The results are in agreement with the previous reports that various solvent extract of Trapa fruit rind showed maximum inhibition activity against gram negative bacteria *Klebsiella pneumonia* and *Pseudomonas testosterone* (Parekh & Chanda 2007). Negative control DMSO did not make any inhibition for all studied strains. Results were compared with the standard antibiotics Gentamicin (25µg/well) and fluconazole (50µg/well). Various plant parts of *Thevetia* such as leaf, seed oil, stem, flower etc were reported as good resources of antimicrobials against a wide array of bacterial and fungal pathogens.

From the present investigation it is understood that various fractions of fruit rind extract possessed inhibitory effect against the entire tested organisms. Published information regarding the *Thevetia* fruit wall extracts was found nil to compare the present results.

Table 1: Comparison of Antimicrobial activity of different array of *T. neriifolia* rind Ethyl Acetate fraction (10mg /50µl/well)

Organisms	Y	O	W	±ve
Gram positive bacteria				Gentamicin
S. aureus	9.00±0.00	0.00±0.00	0.00±0.00	19.67 0.58
S. pyogenes	13.50±0.50	16.83±0.29	13.67±0.42	14.67±0.58
N.asteroids	21.00±0.00	20.00±0.00	21.33±1.15	22.00±0.00
Gram negative bacteria				
P. aeruginosa	19.67±0.58	21.17±1.04	21.67±0.58	29.00±1.00
P.mirabilis	15.50±0.50	17.17±0.76	16.50±0.50	31.00±1.00
Fungal strains				Fluconazole
C.albicans	15.50±0.71	15.50±0.71	15.50±0.71	11.33±0.58
T. rubrum	14.67±0.58	14.67±0.58	14.67±0.58	13.67±0.58

Y- Yellow. O-Orange, W- White

Table 2: Comparison of Antimicrobial activity of different array of of *T. neriifolia* rind Methanol fraction (10mg /50µl/well)

Organisms	Y	O	W	±ve
Gram positive bacteria				Gentamicin
S. aureus	11.17±0.29	11.50±0.50	11.00±0.00	19.67 0.58
S. pyogenes	8.00±0.00	10.33±1.53	8.00±0.00	14.67±0.58
N.asteroids	12.67±0.58	12.67±0.58	11.00±1.00	22.00±0.00
Gram negative bacteria				
P. aeruginosa	12.50±0.50	13.50±1.50	13.50±0.50	29.00±1.00
P.mirabilis	11.67±0.29	11.17±1.04	11.67±0.58	31.00±1.00
Fungal strains				Fluconazole
C.albicans	11.67±0.58	11.67±0.58	11.33±1.53	11.33±0.58
T. rubrum	10.33±0.58	9.00±0.00	9.67±0.58	13.67±0.58

Y- Yellow. O-Orange, W- White

While attempting to compare the sensitivity within the fractions of yellow variant, the sensitivity varies in the following order EA>MT>CH>PE. Comparing the results among EA fractions (Table 1) for better activity, *S. pyogenes* and *P. mirabils* were more susceptible to EA fraction of orange array, and *P. aeruginosa* to white form. Similar results were obtained for MT fraction also for *S.pyogenes*. While evaluating CH fractions, *N. asteroids* and *P.mirabilis* responded well for yellow forms, but among the fungal strains *C. albicans* was more sensitive to white variant (Fig 2). Variations in the antimicrobial sensitivity towards the extracts might be due to differences or variations in phytochemical constituents and the curative properties might reside in these secondary metabolites (Lozoya & Lozoya, 1989, Karthikeyan *et al*, 2009**).** These observations can be rationalized in terms of the polarity of the compound being extracted by each solvent and in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse into the media used in the assay (Parekh & Chanda, 2007). Kouitcheu *et al* (2013) reported the antidiarrhoeal property of fruit rind of *Picralima nitida*, a member of Apocynaceae. Studies conducted by Chanda *et al* (2010) proved that fruit peels and vegetable wastes thrown into the environment as agro waste can be utilized as a source of antimicrobials.

Table 3: DPPH Assay of different fractions of *T. neriifolia* rind (yellow array) extract

Concentration (mg/ml)	PE	CH	EA	MT
0.2	5.438±1.313	11.141±0.000	9.946±0.064	2.957±1.550
0.4	6.366±1.032	16.015±0.141	14.356±2.100	8.472±1.550
0.6	6.101±0.469	19.065±0.328	23.672±0.891	15.681±0.282
0.8	8.952±0.563	27.321±1.125	33.078±0.827	26.346±0.235
1.0	11.638±0.234	33.024±0.750	41.224±1.909	33.156±0.094
1.2	14.125±0.469	41.578±0.188	50.090±0.318	40.266±0.094

PE- Petroleum ether, CH- chloroform, EA- Ethyl Acetate, MT- Methanol

Antioxidant property of the rind extracts were assessed by DPPH scavenging assay. Concentrations ranging from 0.2-1.2 mg/ml were analyzed for all 16 extracts. Bioactive molecules present in the extracts, act as scavengers of free radicals in the test solution. The results revealed the scavenging activity in a doze dependant manner. EA fraction of yellow array showed 50% inhibition in the highest concentration (Table 3), revealed the efficacy of the solvent in extracting maximum antioxidants from 1.2mg of extract to reduce half of dpph molecules. The PE fraction showed least activity with inhibition values 13.46± 0.46 to 15.41±0.51 at 1.2 mg/ml, much better activity was recorded by CH (40.05± 0.28- 43.36± 0.46) and MT fractions (35.61 ± 0.09 to 40.26 ± 0.09), but the EA fraction showed a decrease in activity from 50.09 ± 0.31 to 33.42 ± 0.28 to 27.38 ± 0.46 in the order of yellow, orange and white arrays. Even though the quantity of antioxidants is less when compared to standard ascorbic acid (IC_{50} - 2.5µg/ml), its presence imparts a significant role in traditional system of medicine. Crude extracts compared with extra pure standard antibiotics, reveals the potency of the fractions in terms of active molecules.

CONCLUSION

Antimicrobial activity of three variants of yellow oleander fruit wall was assessed by well diffusion method against seven human skin pathogenic microbes. Ethyl acetate is the most effective solvent to extract many of the active biomolecules that fight against harmful pathogens, support the folklore use of this plant against many skin infections. Although antioxidants present in fruit pulp is not sufficient to cause 50% inhibition in the studied concentrations, there is a considerable potential for the fruit rind in reducing diseases related to free radical mechanisms. Obviously, this plant part is a promising source of antimicrobials, which can be profitably exploited for skin protection. More work, however, is required to develop the extract into a clinically useful antimicrobial and antifungal agent.

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