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EFFECT OF FREE AND BOUND FLAVONOIDS FROM *WRIGHTIA TINCTORIA (ROXB)* R. BR. LEAVES ON SKIN PATHOGENS

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ABSTRACT

The causative agents of skin infection are bacteria, fungi and in specific dermatophytes. Many infectious diseases have been treated with plant extracts. The free and bound flavonoids fractions of *Wrightia tinctoria (Roxb)* R.Br. (Apocynaceae), leaves were tested against 10 bacterial, 3 fungal and 4 dermatophytic strains affecting the skin using disc diffusion and broth micro dilution method. The free flavonoidal extract showed maximum activity against *Bacillus subtilis* (gram positive), *Proteus vulgaris* (gram negative), *Candida albicans* (fungus) and *Trichophyton rubrum* (dermatophyte). The bound flavonoidal extract showed maximum activity against *Staphylococcus aureus* (gram positive), *Escherichia coli* (gram negative), *Candida albicans* (fungus) and *Epidermophyton floccosum* (dermatophytes) among the various tested organisms. The bounded flavonoidal extract (activity index 0.58) had strong activity against dermatophytes. This provides a lead that, flavonoidal extract could be used for direct incorporation into formulations for effective treatment and a scientific rationale for the traditional use of *Wrightia tinctoria* in treating skin infections.

KEYWORDS: **Antibacterial AntifungalAntidermatophytic Flavonoidal extracts*Wrightia tinctoria (Roxb.)* R. Br.**

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INTRODUCTION

Naturally occurring antimicrobials are in great demand globally and plant derived products is considered biodegradable and safe to environment compared to chemically synthesized products.[1.] Herbal medicines have made the large contribution to human healthiness; most of them speed up the natural healing process of human. Herbal medicines have been used in medical practice since antiquity and gained considerable momentum worldwide during the past decade and play foremost role in health care treatments. Dermatology is concerned with the diagnosis and treatment of diseases of the skin, hair and nails. The skin is the largest and most visible organ of the body. It reflects the health of the body and acts as a barrier against injury and bacteria[2]. The skin is a protective covering impenetrable to life threatening microorganisms and resistant to harmful UV rays. The main causative agents of skin infection are bacteria and fungi. Bacteria namely, Bacillus subtilis (causes foot odour), Bacillus cereus (Keratitis), *Micrococcus luteus* (In skin sweat), *Staphylococcus aureus* (folliculitis), *Staphylococcus epidermidis* (epidermatitis), *Escherichia coli* (UTI infection), *Proteus vulgaris* (Intertrigo), *Pseudomonas aeruginosa (*folliculitis), *Salmonella typhi* (cutaneous lesions), *Salmonella paratyphi* (skin lesions and rashes), fungi namely, *Aspergillus flavus* (Aspergillosis), *Aspergillus niger* (Aspergillosis), *Candida albicans* (diaper rash) and dermatophytes namely, *Epidermophyton floccosum* (ring worm), *Microsporum canis (Tinea capitis*), *Trichophyton mentographytes* (Tinea pedis), *Trichophyton rubrum* (Tinea pedis) are some pathogens which cause the mentioned skin infection[3]. Hence they were selected for the present investigation.There is a growing movement to find new medicines, or rediscover old ways of treating illness and improving general health. Flavonoids are phenolic substances widely distributed in the plants. There are a group of about 4000 naturally occurring compounds known to have contributed to human health through our daily diet[4]. They exhibit antimicrobial and other medicinal properties[5]. The wide distribution of antibiotic principles has comprehensively been discussed[6]. Many reports suggest that flavonoids of plants belonging to various families exhibit antimicrobial activity against bacterial and fungal pathogens[7, 8, 9] *Wrightia tinctoria (Roxb)* R. Br. belongs to family Apocynaceae. Its leaves were soaked in coconut oil for few hours and applied for eczema, psoriasis and other skin diseases[10] by ethenic groups in hills. To check the scientific rationale behind the traditional use of leaves of *Wrightia tinctoria* and the role of free and bound flavonoids in it, on its activity against skin pathogens, the present study is carried out. Here specific extracts were prepared from leaves of *Wrightia tinctoria* and screened for their antibacterial, anti fungal and anti dermatophytic activity against skin pathogens.

MATERIALS AND METHODS

1. Collection and authentication of plant material

The leaves of *Wrightia tinctoria* were collected from VInYY garden, Nachallur, Karur district, identified by Prof. P. Jayaraman of Plant Anatomy Research Centre (PARC), Chennai. and a voucher specimen was deposited at PARC.

2. Extraction and fractionation of plant material

The leaves were collected and washed in running tap water to remove adhering dust materials. The cleaned leaves were shade-dried and finely powdered using blender for extraction. The dried and powdered leaves (100g) were soxhlet extracted[11] in 80 per cent methanol (500 ml) for 24 hours on a water bath. The extract was concentrated and re-extracted with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) in succession. The petroleum ether extract was rejected as being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids

while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with 7% H_2SO_4 for 2 hours. The resulting mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate extract thus obtained was neutralized with 5% NaOH. The ethyl ether fraction (free flavonoids) and ethyl acetate fraction (bound flavonoids) were dried in vacuo and weighed. The free and bound flavonoids were re-suspended in their respective solvents to get 10 mg/ml and were used for testing antimicrobial activity against skin pathogens.

3. Antimicrobial activity

For antimicrobial activity 5 gram positive, 5 gram negative, 3 fungal and 4 dermatophytic strains were used.

Test pathogenic microorganisms

Gram-positive bacteria: *Bacillus subtilis (*MTCC 2423), *Bacillus cereus* (MTCC 1305), *Micrococcus luteus* (MTCC 1541), *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 2639).

Gram-negative bacteria: *Escherichia coli* (MTCC 443), *Proteus vulgaris* (MTCC 1771), *Pseudomonas aeruginosa (*MTCC 424), *Salmonella typhi* (MTCC 98), *Salmonella paratyphi* (MTCC 735).

Fungi: *Aspergillus flavus* (MTCC-1783), *Aspergillus niger* (MTCC 1344), *Candida albicans* (MTCC 227).

Dermatophytes: *Epidermophyton floccosum* (MTCC 7880), *Microsporum canis (*MTCC 2820), *Trichophyton mentographytes* (MTCC 7687), *Trichophyton rubrum* (MTCC 3272).

The pathogenic microorganisms were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial strains were grown and maintained on Muller-Hinton Agar medium (HIMEDIA), while fungi and dermatophytes were maintained on Sabouraud Dextrose Agar medium (HIMEDIA).

4. Testing for Anti microbial activity

Disc diffusion method[12] was performed for screening anti microbial activity. Sterile Muller-Hinton agar (for bacterial strains) and Sabouraud Dextrose Agar (for fungal and dermatophytes) base plates were seeded with respective inoculum (inoculum size 1×10^8 CFU/ml for bacteria and 1×10^7 cell/ml for fungi and dermatophytes.) Sterile filter paper discs of Whatmann no.1 (6mm in diameter) were impregnated with 100µl of each of the extract of concentration (10mg/ml) to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in five replicate along with standard antibiotics (positive control) streptomycin (1mg/disc) for bacteria and terbinafine (1mg/disc) for fungi and dermatophytes. The petri plates containing the paper discs (6mm) dipped in ethyl ether, ethyl acetate and 80 percent methanol and water were run parallel to study the impact of the solvent itself (without plant components) on growth of the test organisms. The plates were kept at 4ºC for 45 minutes for the diffusion of extract, thereafter were incubated at 37 ± 2ºC for bacteria (18-24 hrs) and 27 ± 2ºC for fungi (48-72 hrs). Growth was monitored for 24, 48 and 72 hrs, depending on the period of incubation time required for the visible growth. The growth in treated samples was compared with their respective control plates. Inhibition zones formed around the discs were measured with a transparent ruler (in millimeters). The average of inhibition zones was recorded and compared with the standard reference antibiotics. Activity index for each extract was calculated by the standard formula as below:

$$\text{Activity index (AI)} = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}$$

5. *Minimum inhibitory concentration* (MIC) value was determined following Broth micro dilution method[13]. Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100µl inoculums (for bacteria 1×10^8 CFU/ ml and 1×10^7 cell/ml for fungi) were added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37 ± 2ºC for24 h for bacteria and 27 ± 2ºC for 48 h for fungi. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4ºC for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal / fungicidal concentration (MBC/MFC) was determined by sub culturing 50 µl from each well showing no apparent growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC/MFC.

STATISTICAL ANALYSIS

Mean value and standard deviation were calculated for each tested microbe. The data were analysed by one way ANOVA and p values were considered significant at $p>0.005$.

RESULTS

The extraction protocol which has been carried out in the present investigation is exclusively meant for free and bound flavonoids. In the extraction procedure ethyl ether and ethyl acetate fractions are supposed to contain free and bound flavonoids, respectively. Here bound flavonoids imply that the flavonoids are bounded with sugar moiety. These bounded sugar moieties are removed (from ethyl acetate fraction) by acid hydrolysis during extraction. If sugar part of the flavonoids is not removed, extracts do not react with spraying reagents (i.e. 5% fehling solution and 1% $AlCl_3$ solution) during TLC analysis. On the other hand, after removal of sugar part, flavonoids become free from sugars bounded to it and now react with spraying reagent and give colour reactions during TLC analysis. Spraying reagents 5% fehling solution and 1% $AlCl_3$ solution are exclusively used to detect flavonoids[14]. Ethyl ether fraction contains free flavonoids i.e. flavonoids free from sugar moiety and during TLC analysis and thus shows positive colour reactions with the spraying reagents. In the ethyl ether fraction there is no need of acid hydrolysis as flavonoids in this fraction are not bound with sugar.In the present study the free and bounded flavonoids of the *Wrightia tinctoria* leaves were tested for their bioactivity against skin pathogens. The disc diffusion method was used to determine the inhibition zones of extracts (free and bound flavonoids). Both the extracts provided more consistent antibacterial and antifungal activity against all the organisms and especially the bounded flavonoidal extract showed good activity against fungal strains including the dermatophytes. The inhibition zones and activity index are summarized in Table 1. The bounded flavonoids exhibited maximum activity against *Staphylococcus aureus* among the various tested Gram positive bacterial strains (zone of Inhibition 14.2 mm) and minimum activity against *Bacillus subtilis* (zone of inhibition 9.4 mm), whereas the free flavonoidal fraction exhibited maximum activity against *Bacillus subtilis* (zone of inhibition 15.2 mm) and minimum activity against *Staphylococcus aureus* (zone of inhibition 8.6 mm). It is graphically compared with the standard in figure 1. The activity index (which is above 0.4) gives an indication to screen both extracts on other gram positive organisms other than skin pathogens. The bounded flavonoids exhibited maximum activity against *Escherichia coli* among the various Gram negative bacterial strains (zone of

inhibition 14.2 mm) and minimum activity against *Salmonella paratyphi* (zone of inhibition 10.4 mm), whereas the free flavonoidal fraction exhibited maximum activity against *Proteus vulgaris* (zone of inhibition 13.6 mm) and minimum activity against *Pseudomonas aeruginosa* (zone of inhibition 8.2 mm). It is graphically compared with the standard in figure 2. The pronounced activity of bounded flavonoid extract indicated through its activity index (0.65 maximum) prompts wide application feasibility of this extract on other gram negative organisms, other than skin pathogens.The bounded flavonoids exhibited maximum activity against *Candida albicans* among the various fungal strains (zone of inhibition 14.2 mm) and minimum against *Aspergillus niger* (zone of inhibition 9.6 mm), whereas the free flavonoidal fraction exhibited maximum activity against *Candida albicans* (zone of inhibition 7.8 mm) and minimum activity against *Aspergillus flavus* (zone of inhibition 6.6 mm). It is graphically compared with the standard in figure 3. The activity index of bounded flavonoids (max =0.76) encourages its utility as a vital antifungal agent.The bounded flavonoids exhibited maximum activity against *Epidermophyton floccosum* (zone of inhibition 18.6 mm) among the various dermatophytes and minimum against *Microsporum canis* (zone of inhibition 14.2 mm), whereas the free flavonoidal fraction had maximum activity against *Trichophyton rubrum* (zone of inhibition 9.8 mm) and minimum activity against *Epidermophyton floccosum* (zone of inhibition 6.2 mm). It is graphically compared with the standard in figure 4. The bounded flavonoidal extract though had variation in its activity among the various dermatophytes still, the activity index of maximum 0.58 indicated its strong inhibitory property against dermatophytes in comparison to other bacterial and fungal strains. This gives a lead to work on *Wrightia tinctoria* bound flavonoidal extract's activity against other dermatophytic organisms.MIC and MBC / MFC values (Table 2) were evaluated for those organisms against which *Wrightia tinctoria* had an activity index of minimum 0.4 in diffusion assay. The range of MIC and MBC / MFC of extracts recorded was 0.31-2.5 mg/ml. In the present investigation for free flavonoids MIC value was 0.312 mg/ml, and MBC value was 0.625 mg/ml for all the gram positive bacterial strains and *Proteus vulgaris* in gram negative strain, proving the free flavonoidal extract to be bacteriostatic in nature. As its activity index was less than 0.4 for fungal strains it was not subjected for MIC study. For the bounded flavonoidal extract the lowest MIC value, 0.312 mg/ml was observed for all gram positive strains tested except *Bacillus cereus* and the MBC value for all strains were higher than MIC values indicating bacteriostatic effect of the extract. For gram negative organisms the lowest MIC value 0.312 mg/ml was observed for *Proteus vulgaris* and *Pseudomonas aeruginosa* and on comparison of MIC with MBC values, it indicated the bound flavonoidal extract to be bacteriostatic except against *Salmonella paratyphi* for which it was showing bactericidal effect. The least MIC of bounded flavonoids extract against fungal strains was 0.312 mg/ml for *candida albicans* and for all fungal strains it exhibited fungi static effect. High MIC values are indication of low activity while low MIC values are indication of high activity. The MIC of the extracts was not comparable with that of standard antibiotics, possibly because the extract is a crude form and antibiotic was a pure compound. However the extracts expressed bacteriostatic, fungistatic and fungicidal activity against specific organisms indicating their broad spectrum of action. Thus this investigation documents valid evidence that flavonoids which are poly hydroxy phenols have effective antimicrobial activity against a wide range of microorganisms, particularly skin pathogens.

Table 1
Antimicrobial activity of free and bounded flavonoids of Wrightia tinctoria leaf on skin pathogens

Microorganisms	Inhibition zone in diameter (mm)				Activity index	
	Free flavonoid	Bounded flavonoid	Standard	Solvent control	Free flavonoid	Bounded flavonoid
Gram positive bacterial strains						
Bacillus subtilis (MTCC 2423)	15.2 ± 0.84	9.4 ± 0.55	23.4 ± 1.14	-	0.65	0.40
Bacillus cereus (MTCC 1305)	13.6 ± 0.55	10.2 ± 1.30	27.8 ± 0.84	-	0.49	0.37
Micrococcus luteus (MTCC 1541)	08.8 ± 0.45	11.4 ± 0.55	19.6 ± 0.89	-	0.45	0.58
Staphylococcus aureus (MTCC 96)	08.6 ± 0.55	14.2 ± 0.84	20.6 ± 1.14	-	0.42	0.69
Staphylococcus epidermidis (MTCC 2639)	10.8 ± 1.30	12.6 ± 0.89	23.2 ± 0.84	-	0.47	0.54
Gram negative bacterial strains						
Escherichia coli (MTCC 443)	9.4 ± 0.55	14.2 ± 0.84	24.6 ± 0.89	-	0.38	0.58
Proteus vulgaris (MTCC 1771)	13.6 ± 0.89	10.8 ± 0.45	26.6 ± 0.55	-	0.51	0.41
Pseudomonas aeruginosa (MTCC 424)	8.2 ± 0.84	13.4 ± 1.14	20.6 ± 0.89	-	0.40	0.65
Salmonella typhi (MTCC 98)	8.4 ± 0.89	13.2 ± 0.84	22.8 ± 1.10	-	0.37	0.58
Salmonella paratyphi (MTCC 735)	6.8 ± 0.45	10.4 ± 0.55	19.2 ± 0.84	-	0.35	0.54
Fungal strains						
Aspergillus flavus (MTCC-1783)	6.6 ± 0.89	10.2 ± 1.10	21.2 ± 0.45	-	0.31	0.48
Aspergillus niger (MTCC 1344)	6.8 ± 0.84	9.6 ± 0.55	21.2 ± 0.84	-	0.32	0.45
Candida albicans (MTCC 227)	7.8 ± 0.84	14.2 ± 1.30	18.6 ± 0.55	-	0.42	0.76
Dermatophytes						
Trichophyton mentographytes (MTCC 7687)	6.4 ± 0.55	17.6 ± 1.14	30.2 ± 1.10	-	0.21	0.58
Trichophyton rubrum (MTCC 3272)	9.8 ± 0.84	16.2 ± 1.10	31.8 ± 1.30	-	0.31	0.51
Epidermophyton floccosum (MTCC 7880)	6.2 ± 0.45	18.6 ± 0.55	31.8 ± 1.10	-	0.19	0.58
Microsporum canis (MTCC 2820)	6.8 ± 0.45	14.2 ± 0.45	29.6 ± 0.89	-	0.23	0.48

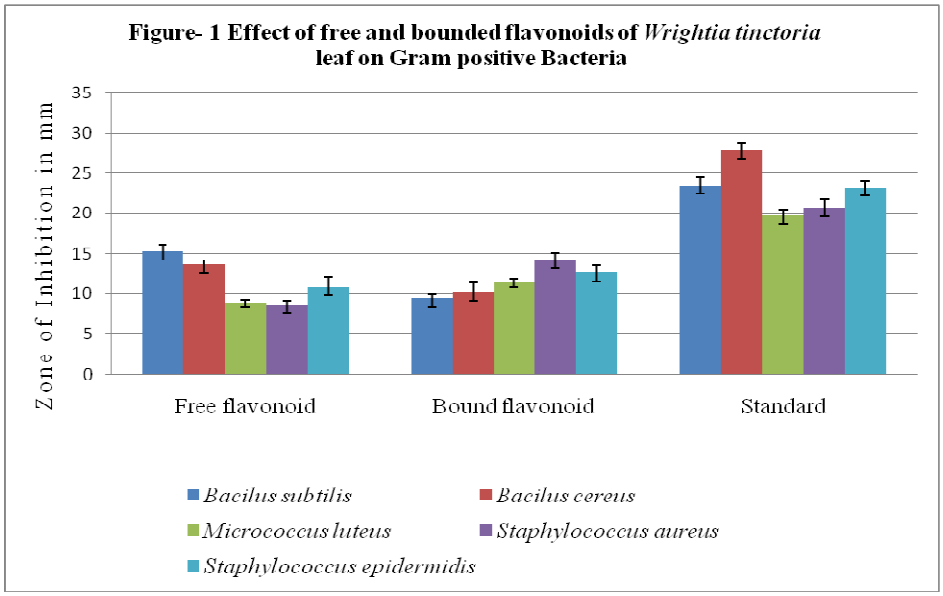

Figure-2 Effect of free and bounded flavonoids of *Wrightia tinctoria* leaf on Gram negative Bacteria

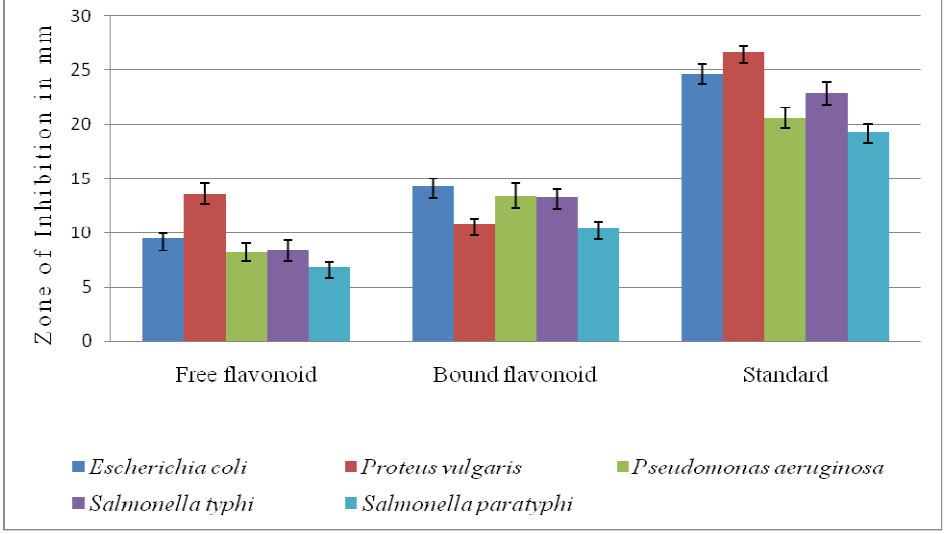

Figure-3 Effect of free and bounded flavonoids of *Wrightia tinctoria* leaf on Fungal strains

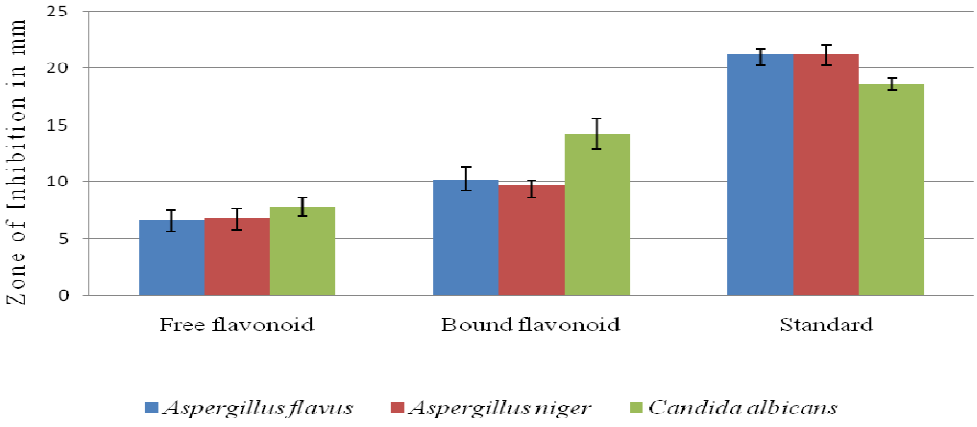

Figure-4 Effect of free and bounded flavonoids of *Wrightia tinctoria* leaf on Dermatophytes

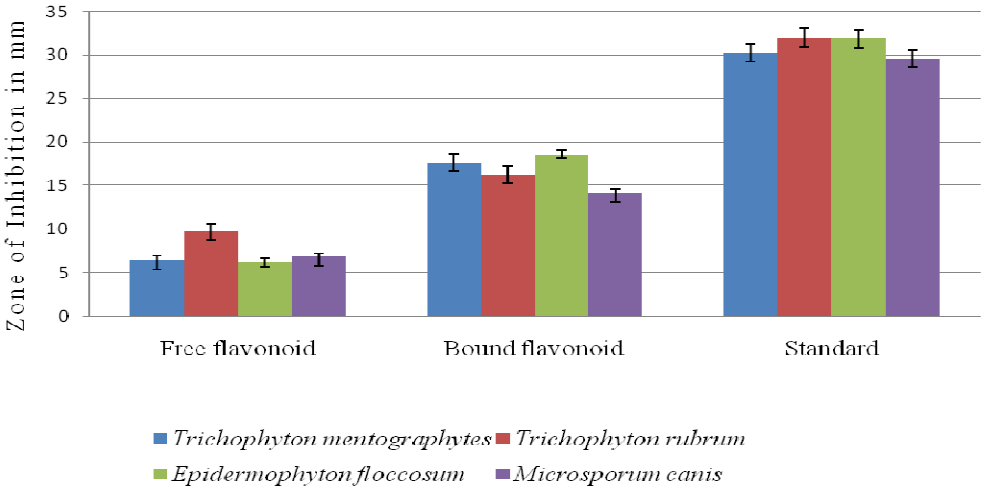

Table 2
MIC and MFC/MBC values of free and bounded flavonoids of Wrightia tinctoria leaf

	Free flavonoid		Bounded flavonoid	
	MIC	MBC / MFC	MIC	MBC / MFC
	mg/ml	mg/ml	mg/ml	mg/ml
Gram positive bacterial strains				
Bacillus subtilis (MTCC 2423)	0.312	0.625	0.312	0.625
Bacillus cereus (MTCC 1305)	0.312	0.625	-	-
Micrococcus luteus (MTCC 1541)	0.312	0.625	0.312	1.25
Staphylococcus aureus (MTCC 96)	0.312	0.625	0.312	0.625
Staphylococcus epidermidis (MTCC 2639)	0.312	0.625	0.312	0.625
Gram negative bacterial strains				
Escherichia coli (MTCC 443)	-	-	0.625	1.25
Proteus vulgaris (MTCC 1771)	0.312	0.625	0.312	1.25
Pseudomonas aeruginosa (MTCC 424)	-	-	0.312	1.25
Salmonella typhi (MTCC 98)	-	-	0.625	1.25
Salmonella paratyphi (MTCC 735)	-	-	0.625	0.625
Fungal strains				
Aspergillus flavus (MTCC-1783)	-	-	0.625	1.25
Aspergillus niger (MTCC 1344)	-	-	0.625	1.25
Candida albicans (MTCC 227)	-	-	0.312	1.25
Dermatophytes				
Trichophyton mentographytes (MTCC 7687)	-	-	0.625	1.25
Trichophyton rubrum (MTCC 3272)	-	-	0.625	2.5
Epidermophyton floccosum (MTCC 7880)	-	-	0.625	1.25
Microsporum canis (MTCC 2820)	-	-	0.625	2.5

DISCUSSION

Wrightia tinctoria is a widely used plant, traditionally in our alternative system of medicinal practice for the treatment of skin infection[15]. Now with the advent of newer antibiotics and new infectious diseases we are observing development of multi drug resistance by organisms to different antibiotics. But *Wrightia tinctoria* is such a plant which is used since time immemorial for skin infection and still effective[15]. The medicinal plants are effective curative agents mainly due to their secondary metabolites synthesized insitu. We have many medicinal plants whose alkaloids, flavonoids, tannins etc that are working as curative agents against many infections. Hence preparations of specific, selective extracts of secondary metabolites, that are more effective in comparison to other solvents extracts of the same plant, against specific micro organisms when determined will be very useful for the following purposes:

1. We can go for formulation using those extracts for curative purpose.
2. We can use them in place of antibiotics against those organisms that are developing resistance.
3. The formulation of these extracts will have better activity than formulation of whole extracts.

Hence the bound and free flavonoidal extracts of *Wrightia tinctoria* were prepared using ethyl acetate fraction and ethyl ether fractions of methanol extract. Anti microbial screening in detail of *Wrightia tinctoria* so far has not been worked out for bound and free flavonoids. Mostly the crude extracts have been screened, but without quantitative analysis on skin pathogens. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence cannot replace the existing antibiotics. In the present study, zone of inhibition, Activity index,

Minimum inhibitory concentration and MBC/MFC have been evaluated for both the extracts against 5 gram positive, 5 gram negative, 3 fungal and 4 dermatophytic strains. The MIC values for many organisms were low for the extracts indicating strong bio antimicrobial efficiency of the plant against skin pathogens.

CONCLUSION

Thus the antimicrobial screening of bound and unbound flavonoidal fractions of *Wrightia tinctoria* against skin pathogens has given us a lead to trial on formulation using them in future and it may be also possible to trace their mechanism of antimicrobial action and thereby locate their suitability as candidates for antibiotics against which, micro organism are developing drug resistance. This study thus advocates the use of selected plant by the pharmaceutical industries for preparing flavonoids based antimicrobial drugs for resistant pathogens.

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