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Possibilities of Developing New Formulations for Better Skin Protection from a Traditional Medicinal Plant Having Potent Practical Usage

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Authors' contributions

This work was carried out in collaboration between all authors. Author AD managed the literature searches. Authors AD, BD and SGD designed the study. Authors JL, AM, SP and DSR carried out the experimental studies and the analyses. Authors BD and SGD wrote the protocol and also the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Natural products obtained from different types of plants have made an important impact since ancient times throughout Asia. Since wood of *Hesperethusa crenulata* (Roxb.) Roem has been used traditionally by women in Southeast Asia for protection against sunlight and other harmful environmental factors, present study was aimed to determine antioxidant and antimicrobial properties of water extract of *H. crenulata* wood.
Methodology: Wood was powdered, soaked in water, macerated, extracted fluid was lyophilized and tested for antioxidant and antimicrobial properties following standard techniques. The IC_{50} values were calculated from regression equations prepared from concentrations of extract and inhibition percent of free radical formation. The extract was

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screened for antimicrobial action following international guidelines against 44 organisms.
Results: In DPPH assay system IC_{50} value was 344.74 µg/ml, the same for superoxide scavenging assay was 326.13µg/ml, while in hydroxyl radical scavenging system it was 999.85 µg/ml. Among 32 strains of staphylococci 10 strains were inhibited at 25 -50 µg /ml level of the extract, 6 strains at 100 µg /ml, 4 at 200 µg /ml and the remaining 12 strains were resistant. Strains of *Bacillus* spp and *Vibrio vulnificus* were rather sensitive to the extract, while *E. coli* and Pseudomonas aeruginosa were resistant. Thus the extract was inhibitory for bacteria known to invade human skin.
Conclusions: The distinctly beneficial properties of the wood of *H. crenulata* indicate its usefulness as a highly active agent for the protection of skin against invasion by microorganisms and free radicals including those that may come from the sunlight.

Keywords: New formulation; skin protection; antioxidant; antimicrobial; hesperethusa crenulata; thanakha.

1. INTRODUCTION

The application of therapeutics originating from plant sources for various types of illnesses in man had been practiced for thousands of years. It is known that traditional medical systems based on plant products are still prevailing among many tribes and communities throughout the world. It may be mentioned here that almost three fourth of modern medicines have plant-based constituents in some form or other. Ayurveda is the holistic alternative science from Asia and is believed to be the oldest form of science of healing, thereby creating the foundation for all others. The main source of medicinal plants in Asia makes the basis of traditional medicines since the inception of human civilization. Thus it is reasonably important to undertake research studies on medicinal plants. Availability of scientific knowledge in support of the curative effect of folk medicine along with identification of a cheap drug of plant origin having intensive therapeutic potentiality combined with minimal adverse effects have become essential to pursue such studies [1,2].

It is known that human skin serves as a protective barrier between the environmental stress and internal organs of the body. In course of time skin gradually shows a sign of aging since it is continuously exposed to the ultraviolet (UV) radiation and also to various harmful substances present in the air [3]. Different free radicals such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are reactive oxygen species (ROS) continuously being formed metabolically in the human body. Such substances have been found responsible for various ailments including aging [4,5]. According to Moon et al [6] UV irradiation not only influences sunburn, but may influence immuno-suppression leading to skin cancer and photo-aging. The ROS bears the potential of subsequent activation of complex signalling pathways followed by induction of matrix metalloproteinases (MMPs) in the skin cells [7]. As the various products obtained from natural sources are being used to treat 87% of all categorized human illnesses [8], plant products may improve the quality of skin by interfering with the UV irradiation [9] and action of free radicals and microbes. The present study describes the beneficial effect of water extract of the wood of *H. crenulata,* (Roxb.) Roem, which is commonly termed in Myanmar as thanakha, in the elimination of free radicals and also different microbes that are normally present on the skin surface, with a probability of causing sun-burn, accelerated aging and infection on the skin. This study further opens up the scopes of obtaining improved medicaments by making new formulations with the help of thanaka.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The wood of *H. cranulata* was finely grinded, 500 gm of which was taken in a beaker of 1 litre capacity and to this was added 500 ml of distilled water. The mixture was macerated by shaking thoroughly at intervals of 2 hr. After 10 hr the fluid was decanted from the top, filtered and finally lyophilized.

2.1.2 Bacteria

A total of 44 bacterial isolates were taken for the study. These included 28 strains of *Staphylococcus aureus,* 4 strains of *S. epidermidis*, 5 strains of *Bacillus* spp; 4 strains of *E. coli,* 1 strain of *Vibrio vulnificus* and 2 strains of *Pseudomonas aeruginosa*. The organisms were identified according to Collee et al [10] and preserved in freeze-dried ampoules [11].

2.1.3 Media

Liquid media were peptone water (PW) containing 1.0% peptone (Oxoid, UK) plus 0.5% Analar NaCl and nutrient broth (NB, Oxoid). Solid media were nutrient agar (NA, Oxoid) and Mueller Hinton agar (MHA, Oxoid). The pH of all the media were maintained at 7.2-7.4. Gram positive bacteria were grown in NB while Gram negative organisms were cultivated in PW. Antimicrobial sensitivity was determined in both NA and MHA [11,12].

2.2 Methods

2.2.1 Detection of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the extract

The stable DPPH activity of the extract was determined following the method of Braca et al [13]. Aqueous extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm wave length in a spectrophotometer (Varian) was determined after 30 min, and the percent inhibition activity was calculated as [(Ao-Ae)/Ao] x100 (Ao=Absorbance without extract; Ae=absorbance with extract). Increasing amounts of the extract were allowed to react with DPPH and the percentage inhibition of free radicals was recorded. In case of a positive test the colour of the reaction mixture changed from yellow to purple coupled with a simultaneous decrease in the values of absorbance.

2.2.2 Detection of the superoxide radical scavenging activity of the extract

The method used by Martinez et al [14]. for determination of superoxide dismutase as modified by Dasgupta and De [15] was followed in the riboflavin-light-nitrobluetetrazolium (NBT) system [16]. Each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) in 1 ml of a sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm (Varian UV-VIS spectrophotometer) after 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance values of the control and those of the reaction mixture containing the sample solution.

2.2.3 Determination of hydroxyl radical scavenging activity

According to Vasquez-Vivar et al. [17] hydroxyl radicals can be generated by direct addition of iron(II) salts to a reaction mixture containing phosphate buffer. It is known that benzoate is hydroxylated to hydroxybenzoates and also that benzoate after monohydroxylation forms highly fluorescent products [18]. A mixture containing 0.2 ml sodium benzoate (10 mM), 0.2 ml of $FeSO_4,7H_2O$ (10 mM) and EDTA (10 mM) was first prepared in a screw capped bottle. To this mixture were given phosphate buffer (pH 7.4, 0.1 ml) along with the sample solution in order to give a total volume of 1.8 ml. Finally, 0.2 ml of an H_2O_2 solution (10 mM) was added to the above. The reaction mixture was then incubated at 37ºC. After 2 h the fluorescence was measured at 407 nm emission (Em) and 305 nm excitation(Ex) using the spectrofluorimeter (Hitachi). The OH-scavenging activity was expressed as follows [%] = [1-(FIs-FIo)/(FIc-FIo)]x100 where FIo: fluorescence intensity at Ex 305 and Em 407 nm without any treatment, FIc : fluorescence intensity at Ex 305 and Em 407 nm of treated control, FIs : fluorescence intensity at Ex 305 and Em 407 nm of treated sample [19]. In the test proper, the amount of the extract was increased gradually up to 3000 µg/ml amount and the percentage inhibition of formation of hydroxyl radicals in the treated samples was measured as described above.

2.2.4 Determination of IC_{50} value

IC_{50} values (concentration of sample required to scavenge 50% of any type of free radical) were calculated from the regression equations prepared from the concentrations of the extracts and inhibition % of free radical formation in different systems of assay e.g. DPPH assay, superoxide radical scavenging assay and hydroxyl radical scavenging assay. There is an inverse relationship between IC_{50} value and concentration of the sample in any of the assay systems employed in this study.

2.2.5 Determination of minimum inhibitory concentration (MIC) of the extract

The lyophilized powder was added at concentrations of 0 (control), 10, 25. 50, 100, 200 and 400 µg/ml to molten NA/MHA and poured in to Petri dishes according to CLSI guidelines [20]. The test organisms were grown in PW/NB for 18 hr and harvested during the stationary growth phase. A direct suspension of each organism was prepared in 5 ml sterile distilled water. The turbidity of the suspension was adjusted to 0.5 McFarland's standard [21] with a spectrophotometer (Chemito UV 2000 Double beam UV-VIS Spectrophotometer, Mumbai, India) at 625nm, which corresponded to $2.4x10^8$ colony forming units (CFU)/ml. The inocula were prepared by further diluting the suspension 1:100 with sterile distilled water in such a manner that a 2 mm diameter loopful of a culture contained 10^5 CFU. These were then inoculated on the plates containing increasing amounts of the extract along with the control. Readings were taken after 24 hr incubation at 37ºC and thereafter 72 hr. Lowest concentration of the agent that failed to exhibit visible growth was taken as its MIC [20,22]. The entire experiment was performed in triplicate and repeated when necessary.

3. RESULTS AND DISCUSSION

3.1 Result

3.1.1 DPPH scavenging property of the extract

In the determination of the free radical (DPPH) scavenging activity of the extract, it was found that as the amount of the extract was increased there was a steady rise in the

inhibition percent of free radicals Fig. 1. The IC_{50} value with the help of five concentrations of the extract was found to be 344.75 µg /ml and the correlation coefficient (r) was calculated to be 0.9815 (p = 0.001).

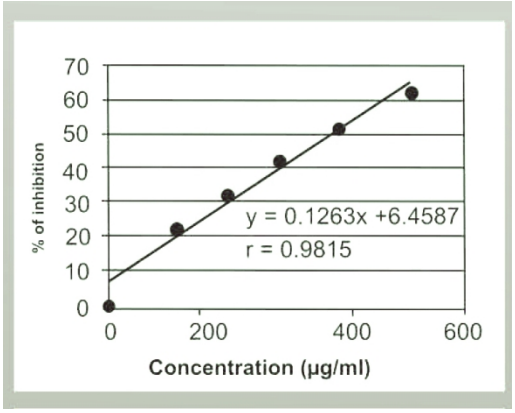

Fig. 1. DPPH radical scavenging activity of the extract

3.1.2 Action of the extract on removal of superoxide

As the amount of extract was increased from 200 µg to 1000 µg, there was a definite gradual increase in the inhibition percent of formation of the superoxide radical Fig. 2, proving thereby the dose-dependent activity of the extract, the calculated values being r = 0.9153 and p =0.001. The IC_{50} value was determined to be 326.13 µg /ml.

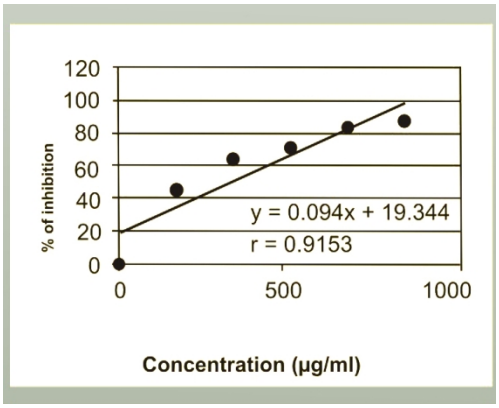

Fig. 2. Superoxide redical Scavenging activity of the extract

3.1.3 Hydroxyl radical scavenging action of the extract

It was observed that with the increase in the amount of extract in the mixture there was a definite decrease in the values of fluorescence. There was a linear correlation (r=0.9256 , p=0.001) between concentrations of the extract and percent of inhibition IC_{50} value was found to be 999.85 µg /ml. Thus this extract revealed as a reasonably good scavenger of the hydroxyl radical Fig. 3.

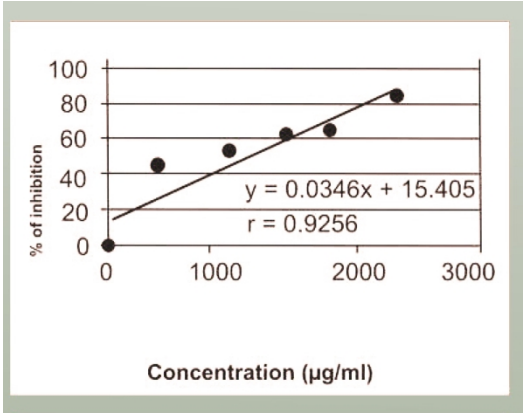

Fig. 3. Hydroxyl redical scavenging activity of the extract

3.1.4. Bacterial inhibitory spectrum of wood extract of H. crenulata

The extracted material was found to be active against many of the test organisms Table. 1. Two strains each of *S. aureus* and *S. epidermidis* and one strain of *B. subtilis* were inhibited at 25 µg/ml of the extract, 4 strains of *S. aureus*, 2 strains each of *S. epidermidis and Bacillus spp.* failed to grow at 50 µg/ml level, 6 other strains of *S. aureus*, 2 strains of *Bacillus* spp. And the single strain of *V. vulnificus* exhibited failure of growth at 100 µg/ml level, The MIC of extract in 4 strains of *S. aureus* was recorded at 200 µg/ml level. Six different *S. aureus* strains had the MIC value at 400 µg/ml, while the remaining bacteria including 4 different strains of *E. coli* and 2 *P. aeruginosa* were resistant to the extract Table. 1.

Table 1. *In vitro* antimicrobial activity of the wood extract of H. crenulata on gram positive and gram negative bacteria

Bacteria	No	MIC of wood extract	
		tested	(µg/ml)
Staphylococcus aureus	ML264, ML322	2	25
S.aureus	ML81, ML83, NCTC8530, NCTC8531	4	50
S.aureus	WS2, ML6, ML19, ML117, ML177, ML420	6	100
S.aureus	ML2, ML118, ML139, ML318	4	200
S.aureus	ML149, ML185, ML267, ML329, ML345, ML411	6	400
S.aureus	ML17, ML162, ML169, ML191, ML311, ML328	6	>400
S.epidermidis	PH2, PH9	2	25
S.epidermidis	BVC14, BVC23	2	50
Bacillus subtilis	UC564	1	25
B.pumilus	NCTC8241	1	50
B.brevis	NCTC7096	1	50
B.polymyxa	NCTC4747	1	100
B.licheniformis	NCTC10341	1	100
Escherichia coli	55, R244, V517, K12 Row	4	>400
Vibrio vulnificus	A1	1	100
Pseudomonas aeruginosa	ATCC27853, APC1	2	>400
Total no. tested		44	

MIC, minimum inhibitory concentration

4. DISCUSSION

Plants are known to contain a very large number of substances, many of which can be easily extracted. Among the variety of substances, certain plant products may turn out to be rich in antioxidants. Hence such plant products may be considered as promising sources of therapeutic drugs for removal of free radicals. It has been established that exposure of human skin to ultraviolet radiation can induce the expression of MMPs which is known to degrade the extracellular matrix leading to premature aging. Thus recognition of comparatively less harmful plant-derived substances possessing antioxidant properties may prove to be a highly useful strategy for prevention of photo-aging [23].

The antioxidant action of the crude extract of *H. crenulata* wood (without the bark) could be assayed with the help of several test systems. According to Schlesier et al [24] as there are differences in the test systems for determination of this activity, two methods can be recommended without any reservation. The hydrogen-radical scavenging action is a known valuable mechanism of antioxidation and DPPH is a stable free radical. An efficient antioxidant on interaction with DPPH is able to transfer electron of the hydrogen atom to DPPH thereby neutralizing its free radical character [25]. This reducing power is possibly due to the hydrogen-donating ability [26] and is generally associated with the presence of reductones [27]. In our study we found that the aqueous wood extract quenched DPPH free radical in a dose dependant manner Fig. 1.

Superoxide radical has been recognized as a very harmful agent to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals [28]. Moreover, a superoxide radical is considered to play an important role in the peroxidation of lipids [29].

During the course of normal oxidative phosphorylation, between 0.4 – 4% of all oxygen consumed is converted into free radical $O_2^{-\cdot}$[30]. Xanthine oxidase (XOD) mediates generation of $O_2^{-\cdot}$ during oxidation of hypoxanthine to uric acid and Molecular oxygen acts as the electron acceptor during the reoxidation of XOD to generate $O_2^{-\cdot}$ [31]. Functioning of immune system such as phagocytosis stimulates activation of NADPH oxidase, an enzyme normally inactive in resting cells and production of O_2^- [32] is then converted to H_2O_2 by superoxide dismutase. Hydroxyl radicals are generated by Fenton reaction in presence of reduced transition metals such as Cu and Fe and by Haberweiss reaction. Among these radicals $^{\cdot}OH$ is the most reactive [33]. These oxygen radicals may induce some oxidative damage to biomolecules [34,35]. During the present study it has been observed that the crude aqueous extract of the wood of *H. crenulata* scavenged both the radicals, $O_2^{-\cdot}$ and OH in a dose dependent manner Figs. 2 and 3. Such activities are likely to prevent damage of biomolecules due to oxidative stress and aging.

The antimicrobial activity revealed concentration-dependent nature of the extract; with the increase in the amount of the extract there were growth failures among the test bacteria. Both *S. aureus* and *S. epidermidis* not only comprise normal flora of human skin but are also capable of causing various types of skin infections as the opportunity arrives. Although *Bacillus* spp can occasionally invade skin, several Gram negative bacteria including *Vibrio vulnificus, E. coli* and *Pseudomonas aeruginosa* are able to cause mild to severe infections of the abraded skin is bedsores. Our observation on resistances of Gram negative organisms to the extract may be explained due to the fact that these bacteria possess an outer lipid membrane which can act as a barrier to many environmental substances including antibiotics [6,11,12]. This extract is essentially moderate to highly active against many

possible invaders of human skin. Thus the present study reveals that the crude extract of *H. crenulata* wood can be claimed as a material with a high potentiality of being used for protection of the skin not only against free radicals but also against microbial invaders of the skin.

It needs to be mentioned here that this particular plant grows throughout the entire country of Myanmar. It is an old and common traditional practice of most women of that country to rub a piece of wood of this plant, termed as thanakha with water over a plate of stone and apply the dough on the face and neck for protection against sunlight as well as other harmful substances present in the air that may be playing a direct or an indirect role in photo-aging. It may be mentioned here that, Joo et al. [36], reported isolation of the UV absorbent compound marmesin from the bark of *H. crenulata*. Their NMR data showed a structure of 2,3—dihydro-2(1-hydro-1-methylethyl)-furanocoumarin, which contained UV absorbing chromatophores. Moreover, plant derived secondary metabolites such as coumarins are known to possess potent antibacterial action [37]. These reports along with our own observation on the antimicrobial and free radical scavenging properties of the water extract of *H. crenulata* wood confirm the suitability of the age old practice of application of thanakha by Myanmar women. In this way a completely new product may be developed by supplementing the extract from thanaka wood in routine natural or synthetic medications for a better and much improved formulation for the skin of women. A rather simple formulation can be made by obtaining a mixture of the extract with emulsifying wax, white soft paraffin and liquid paraffin, or alternatively by combining with water soluble base like polyethylene glycol [38]. However, standard toxicity tests would be required for marketing the new formulation. Such simple formulations can be recommended as these medicaments can be removed from the skin simply with the help of water. Further studies are in progress to make new formulations by adding known natural skin care materials.

5. CONCLUSION

It may be concluded from the study that the wood of *H. crenulata* contains highly beneficial components that can protect human skin from the invasion of microorganisms present in the air and also reactive oxygen species coming from the rays of sun.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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