Evaluation of antioxidant and antiacne properties of terpenoidal fraction of Hemidesmus indicus (Indian sarsaparilla)

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Citation

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Abstract

The present study was undertaken to evaluate the effect of terpenoidal fraction of the roots of Hemidesmus indicus roots (TFHI) on several in vitro antioxidant and antiacne activity. The in vitro antioxidant potential of TFHI was evaluated by various antioxidant assays, including DPPH radical scavenging, superoxide radical scavenging in riboflavin/light/ NBT, and nitric oxide (NO) scavenging activity in sodium nitroprusside/Greiss reagent system. Antiacne activity of TFHI was tested by disc diffusion and broth dilution methods against Propionibacterium acnes and Staphylococcus epidermidis which have been recognized as pus-forming bacteria triggering an inflammation in acne which are etiologic agents of acne vulgaris. Further, preliminary phytochemical analysis and TLC finger print profile of TFHI was established to characterize the terpenoidal fraction which showed antioxidant and antiacne properties. The TFHI was found to have different levels of antioxidant properties in the models tested. In scavenging DPPH and superoxide radicals, its activity was intense (EC50 = 18.7 and 19.3 µg/ml respectively) while scavenging NO radical was moderate. Based on a broth dilution method, the TFHI had the greatest antiacne properties. The MIC values were the same 38 µg/ml for both bacterial species and the MBC values were 38 and 46 µg/ml against Propionibacterium acnes and Staphylococcus epidermidis respectively. The antioxidant and antiacne activity could be attributed to non-polar terpenoidal fraction.

INTRODUCTION

Hemidesmus indicus (L.) Schult. (Asclepiadaceae) is a twining shrub which has been used as folk medicine and as ingredient in Ayurvedic and Unani preparations against disease of biliousness, blood diseases, diarrhea, skin diseases, respiratory diseases, fever, syphilis, bronchitis, eye diseases, loss of appetite, burning sensation rheumatism and gastric disorders [1, 2, 3]. It has also been used in combination with other drugs for snake bite [4, 5]. Recently, this plant was used to treat viper venom {haemotoxic} - induced lethality [6] Ethanolic extract of root was found to be Antihepatotoxic [7] and against ulcer [8]. Methanolic root extracts are proved to have Antidiarrheal [9, 10], antioxidant activity [11]. Renoprotective effect of Hemidesmus indicus is used in gentamycin induced renal toxicity [12]. Some important chemical constituents of the root include hemidesmin1, hemidesmin 2, -amyrin, -amyrin, lupeol, 2-hydroxy—methoxy-benzoicacid and some triterpenes [13, 14]. From the aerial parts of the plant several pregnan steroids have been isolated [15].

Acne vulgaris is a most common skin disorder of Pilosebaceous unit. That affects areas containing the largest oil glands, including the face, back, and trunk [16]. It is generally characterized by formation of seborrhea, comedone, inflammatory lesions and presence of bacteria Propionibacterium acnes, Staphylococcus epidermidis and Malassezia furfur in the follicular canal and sebum production [17]. The role of free radicals in many disease conditions has been well established. In the recent years the areas which attracted a great deal of attention is antioxidants in the control of degenerative diseases in which oxidative damage has been implicated. Several plant extracts and different class of phytoconstituents have been shown to have antioxidant [18, 19] and antiacne activity [20].

In the present paper, we report our work on establishing antioxidant activity of terpenoidal fraction of Hemidesmus indicus (TFHI), in quenching free radicals viz., DPPH,

Superoxide and nitric oxide radicals that are generated in several in vitro models and antiacne activity by disc diffusion and broth dilution method. In the past fe years high performance thin layer chromatography (HPTLC) has emerged as a potential for rapid and efficient phytochemical evaluation of herbal drugs [21], [22]. TLC fingerprinting profile is also established using HPTLC to characterize the extract which would facilitate the identification of TFHI.

MATERIALS AND METHODS

PLANT MATERIAL

Hemidesmus indicus roots were collected in month of October 2005 from tribal area of Western Ghats, Karnataka state India. Authentication of the plant material [RRI/BNG/IDNO./2005/37] was done by comparison with plant specimen located at Bangalore. Herbarium and Botanical Section of Regional Research Institute (Ayurveda), Jaynagar, Bangalore.

PREPARATION OF EXTRACT AND TERPENOIDAL FRACTION

Plant material was dried at 40°C, with forced ventilation, during 3 days, before being powdered and sieved. Only material between 0.210-0.350 mm sizes was utilized. Plant material (50 g) was macerated with the solvent indicated for each species, at the ratio of 1:10 (w/v), room temperature, during 7 days. When necessary, the extracts obtained were fractionated, as described as follows. The terpenoidal fraction which contained mainly triterpenes and/or steroids was obtained by silica gel open-column preparative liquid chromatography. [23]. Terpenoids was separated out and dried under vacuum (25% w/w) Vacuum-dried terpenoidal rich fraction of Hemidesmus indicus (TFHI) was taken for further studies.

PHYTOCHEMICAL TEST AND TLC FINGER PRINT PROFILE OF TFHI

Obtained TFHI was subjected to Liebermann Burchard test for terpenoids [24]. In brief, a small quantity of the TFHI was dissolved in chloroform and treated with acetic anhydride and few drops of concentrated sulfuric acid, which gives a dark pink to red colour, indicating the presence of terpenoids, which were further separated on a thin-layer chromatography (TLC) plate and detected by treatment with anisaldehyde sulfuric acid reagent. TLC finger printing was established for TFHI using HPTLC. A stock solution of [1 mg/ml] of TFHI was prepared in chloroform. Suitable diluted stock solution was spotted on precoated plates of silica gel G60 F254 (E-Merck) using CAMAG Linomat IV automatic sample spotter and the plates were developed on solvent system of different polarities to resolve components of TFHI. The plates were dried at room temperature and scanned using CAMAG TLC scanner III at 254nm (absorbance/reflectance mode) and 366 nm (fluorescence/reflectance mode) and R_f values, spectra and peak areas of the resolved bands were recorded. Relative peak areas of each of the bands were calculated from peak areas. The TLC plates were derivatised by spraying with anisaldehyde Sulphuric acid reagent at 110° C for 5 mins for detection of terpenoids.

FREE RADICAL SCAVENGING ACTIVITY

ASSAY FOR ANTIRADICAL ACTIVITY

Antiradical activity was measured by a decrease in absorbance at 517 nm of a methanolic (MeOH) solution of coloured 1,1-diphenyl-2- picryl hydrazyl (DPPH) was determined by the method described by Braca et al., [25]. TFHI (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was determined after 30 min, EC_{50} and the percentage inhibition activity was calculated from $[(A–A_1)/A] X 100$, Where, A is the absorbance of the control, and A_1 is the absorbance of the TFHI/ standard.

ASSAY FOR SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The assay was based on the capacity of the TFHI to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin–light–NBT system [26]. Each 3ml reaction mixture contained 50mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, and 12mM EDTA, and 0.1 mg NBT and 1ml TFHI solution. Reaction was started by illuminating the reaction mixture with different concentrations of TFHI (25–100 mg/ml) for 90 sec. immediately after illumination; the absorbance was measured at 590nm. 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 anion generation was calculated.

ASSAY OF NITRIC OXIDE SCAVENGING ACTIVITY

The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using

Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations TFHI in methanol and incubated at room temperature for 150 min. The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm. [27]

ANTIACNE TESTING

DISC DIFFUSION METHOD

This experiment was performed by the method [20], Propionibacterium acnes was incubated in brain heart infusion medium (BHI) with 1% glucose for 48 h under anaerobic conditions and adjusted to yield approximately 1.0×10^8 CFU/ml. Aliquots of molten BHI with glucose agar were used as an agar base. A prepared inoculum was added to molten agar, mixed and poured over the surface of the agar base and left to solidity. A sterile paper disc was impregnated with TFHI (25, 50 75 and 100 mg/ml), Clindamycin as standard 10 µg/ml) and the disc was placed on the agar. Plates were then incubated at 37 °C for 48 h under anaerobic conditions in anaerobic jar (Hi-Media) with gas pack and indicator strip and the jar was kept in incubator for 48 h at 37 ± 1°c. Gas packs containing citric acid, sodium carbonate and sodium borohydride were used to maintain and check the anaerobiasis, where citric acid releases carbon dioxide and sodium borohydride releases hydrogen when they come in contact with oxygen. An indicator strip of methylene blue is introduced into the jar which changes the colour from white to blue in absence of anaerobiasis. Staphylococcus epidermidis was incubated in tryptic soy broth (TSB) for 24 h at 37 °C and adjusted to yield approximately 1.0×10^8 CFU/ml. The procedures were the same as mentioned above except the plates were incubated at 37°C for 24 h under aerobic conditions. All disc diffusion tests were performed in three separate experiments and the antibacterial activity was expressed as the mean of inhibition diameters (mm).

DETERMINATION OF MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATIONS

The minimal inhibitory concentration (MIC) values were determined by broth dilution assay [20]. The cultures were prepared at 24 h and 48 h broth cultures of Staphylococcus epidermidis and Propionibacterium acnes, respectively. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.

3 ml of the Nutrient yeast glucose broth (NYG) for Propionibacterium acnes, and Nutrient broth for Staphylococcus epidermidis, in 10 ml glass screw cap test tube was sterilised by autoclaving at 121°c for 15 minutes. The medium was cooled and inoculated with 50 µl of the bacterial suspension containing 1 x 10^8 cells/ml. 1 ml of TFHI (10 mg/ml), was added to corresponding test tubes under anaerobic condition. 3 ml of NYG broth inoculated with 50 µl of organisms was taken as positive control were placed in anaerobic condition at 37 ± 1° c for 48 h. For Staphylococcus epidermidis the test tubes were incubated at 37 ± 1° c for 24 h aerobically and growth of Propionibacterium acnes and Staphylococcus epidermidis was measured as function of turbidity at 660 nm using (Systronics 131) Nephaloturbidometer. The MIC and MBC values TFHI against Propionibacterium acnes and Staphylococcus epidermidis. The results are shown (Table 2) as average values from three separate experiments

RESULTS

Preliminary chemical examination of TFHI showed an intense pink colour with the Liebermann Burchard test, indicating rich terpenoids content in the plant, which was then detected on a TLC plate by treatment with anisaldehyde sulfuric acid reagent and also characterized phytochemically by establishing its TLC finger printing profile. The TLC fingerprint profile comprises of the bands resolved, R_f values, spectral details and max

When scanned in UV 254 and 366 nm. Derivatisation of the TLC plates with 5 % methanolic anisaldehyde sulfuric acid reagent. The details of TLC fingerprinting profile are given in [Table 1] and the chromatogram in [Figure 1].

Figure 1

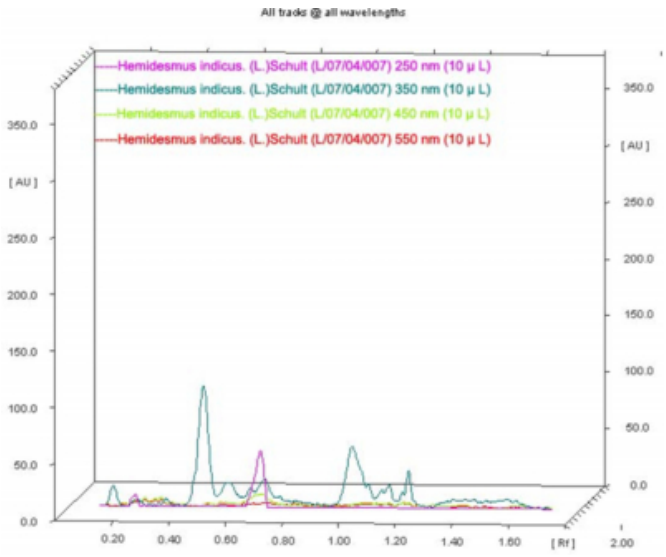
Table 1: TLC fingerprinting profile of terpenoidal fraction of roots [TFHI)

Scanned at	Solvent system I			Solvent system II		
	R_f	λ max	Relative %	R_f	λ max	Relative %
254 nm	0.30	243, 267	12.58	0.16	256, 288	18.14
	0.36	292	10.23*	0.35	285	5.69
	0.62	256,310	13.34	0.41	247	12.87*
	0.77	279	58.78*	0.65	279	61.78*
	0.86	288	3.13 *	0.71	288, 291	6.45*
				0.91	245	2.36
366 nm	0.05	288	6.47	0.18	244	5.87
	0.11	318	7.45	0.24	257	5.79
	0.16	296	20.76	0.65	264,339	49.46
	0.24	281	54.23	0.87	248	15.64
	0.29	308	16.21	0.91	294	16.21
	0.35	303	2.43	0.95	305	2.56
	0.62	291	8.93			

*Bands which showed positive terpenoidal compounds with methanolic anisaldehyde sulphuric acid reagent. ** All bands are fluorescent. Solvent system I- Toluene :Ethyl acetate: Methanol : Acetic acid (7.5:1.5:0.5:0.5 v/v), Solvent system II-Hexane :Ethyl acetate (8:2 v/v)

Figure 2

Figure 1: Densitometric scan at multiple wavelengths of Terpenoidal fraction of roots[TFHI]



ANTIOXIDANT ACTIVITY

The TFHI exhibits different levels of antioxidant activity in all the models studied. It showed a concentration dependant antiradical activity by inhibiting DPPH radical with an EC_{50} value of 18.87 µg/ml (Table 2). The TFHI scavenged superoxide radicals in a dose dependent manner with EC_{50} value of 19.67 µg/ml, which is comparable to the activity ascorbic acid EC_{50} value of 17.19 µg/ml (Table 3). The TFHI also showed moderated nitric oxide scavenging activity also in a dose dependent manner with EC_{50} value of 43.80 µg/ml. (Table 4).

Figure 3

Table 2: Antiradical activity of TFHI observed with DPPH

Sample	Concentration (µg/ml)	% inhibition*	EC_{50} (µg/ml)
TFHI	04.17	08.51 ± 0.22	
	08.33	21.82 ± 0.41	
	16.67	46,71 ± 0.33	18.87
	25.00	66.58 ± 0.50	
	33.33	84.72 ± 2.79	
Ascorbic Acid			01.62

N = 3 * Mean ± SD

Figure 4

Table 3: Superoxide scavenging activity of TFHI observed with a riboflavin-light-NBT system

Sample	Concentration (µg/ml)	% inhibition*	EC_{50} (µg/ml)
TFHI	16.67	43.97 ± 3.70	
	33.33	74.78 ± 5.84	
	50.00	92.95 ± 3.33	19.67
	66.67	98.88 ± 2.56	
Ascorbic Acid			17.19

N = 3 * Mean ± SD

Figure 5

Table 4: Nitric oxide scavenging activity of TFHI

Sample	Concentration (µg/ml)	% inhibition*	EC_{50} (µg/ml)
TFHI	10.00	10.81 ± 1.32	
	40.00	47.75 ± 3.80	
	80.00	71.47 ± 5.95	43.80
	125.00	81.08 ± 3.01	
	150.00	91.59 ± 2.27	
Curcumin			18.47

N = 3 * Mean ± SD

ANTIACNE ACTIVITY

TFHI showed maximum antiacne activity the MIC values were the same (38 µg/ml) for both bacterial species and the MBC values were 38 and 46 µg/ml against Propionibacterium acnes, and Staphylococcus epidermidis respectively. (Table 5).

Figure 6

Table 5: The MIC and MBC values of TFHI

Plant extracts	Susceptibility of bacteria to medicinal plant extract*			
	Propionibacterium acnes		*Staphylococcus epidermidis*	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
TFHI	38	38	38	46
Clindamycin	15	15	15	16

* The results indicate of average of 3 separate experiments

DISCUSSION

Free radicals have been implicated in many disease conditions, the important ones being superoxide radicals, hydroxyl radicals, peroxyl radical and singlet oxygen. Herbal drugs containing radical scavengers are gaining importance in treating such diseases.. Many plants exhibit efficient antioxidant and antiacne activities owing to their terpenoidal constituents [28, 29]. In the present study, terpenoidal fraction of Hemidesmus indicus was investigated the antioxidant and antiacne activities and the possible mechanism involved, basing on the response obtained in different in vitro models covering major free radicals viz., superoxide, hydroxyl and nitric oxide radials and antibacterial against Propionibacterium acnes and staphylococcus epidermidis which are pathogenic factors for acne vulgaris. Further, the TLC fingerprinting for TFHI was established which is responsible for antioxidant and antiacne activities.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule generally used for testing preliminary radical scavenging activity of a compound or plant extract or a fraction. In the present study THFI showed good antiradical activity by scavenging DPPH radical. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, terpenoids, tannins, and aromatic amines reduce and decolourise DPPH by their hydrogen donating ability [29]. Terpenoidal fraction of Hemidesmus indicus are probably involved in their antiradical activity.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species. TFHI was found to be an efficient scavenger of superoxide radical generated in riboflavin-NBT-system in vitro and its activity is comparable to that of ascorbic acid. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. [30]. The

Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- are very reactive. These

compounds are responsible for altering the structural and functional behaviour of many cellular components. Incubation of solutions of sodium nitroprusside in PBS at 25 1°C for 2 h resulted in linear time-dependent nitrite production, which is reduced by the tested methanol extracts. TFHI showed a moderate nitric oxide scavenging activity when compared to DPPH radical and super oxide radical. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions 30.

Propionibacterium acnes and Staphylococcus epidermidis which have been recognized as pus-forming bacteria triggering an inflammation in acne which are etiologic agents of acne vulgaris. Propionibacterium acnes have been described as an obligate anaerobic organism. It is implicated in the development of inflammatory acne by its capability to activate complements and by its ability to metabolize sebaceous triglycerides into fatty acids, which chemotactically attract neutrophils. Staphylococcus epidermidis, an aerobic organism, usually involves in superficial infections within the sebaceous unit. These factors provide a potential target for treatment. Propionibacterium acnes and Staphylococcus epidermidis are the target sites of antiacne drugs [31, 32]. TFHI showed a potent antiacne activity. Many diterpenoids isolated from the Rabdosia plants taste bitter and exhibit various biological activities such as antitumour, antimicrobial and insect growth inhibitory activities. Rosthornins a terpenoid were found to exhibit moderate antibacterial activity. Terpenoids like linalool, nerolidol, geraniol, menthol, borneol, 4-terpineol, 1-octanol, -terpineol and crinitol have showed antiacne activity against Propionibacterium acnes [33, 34]..

CONCLUSION

In conclusion, from the above investigation, using several in vitro antioxidant and antiacne models. Terpenoidal fraction of Hemidesmus indicus [TFHI] root bark was found to scavenge DPPH radical, superoxide radical and nitric oxide radial. TFHI also showed potent antiacne activity against Propionibacterium acnes and Staphylococcus epidermidis. TFHI was characterized for the presence of terpenoids by TLC finger printing profile. The free radical scavenging property may be one of the mechanisms by which the drug is

effective in acne vulgaris and in traditional medicines.

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