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Original Research

Estimation of Mineral Composition, Antioxidant, Antimicrobial, Biofilm Activity and HPLC Profile of *Halothamnus auriculus*

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Abstract

This study focuses on the investigation of antimicrobial activity, essential oil composition, antioxidant potential, phenolic profile, biofilm activity, cytotoxicity and mineral composition from the stem extracts of *Halothamnus auriculus*. The extraction was done using different organic solvents of increasing polarity. The antioxidant potential of all extracts was assessed by DPPH (2, 2-dipheny-1-picrylhydrazyl) radial scavenging ability. The DPPH inhibition (%) was in the range of 49.45-14.84. Cytotoxicity (%) was analyzed by hemolytic activity and it was in the range of 11.90-48.43. Antimicrobial activity was estimated by agar well diffusion method and inhibition zone were measured. Ethanol extract manifested maximum inhibition zone range which was 18-4 mm against bacterial strains *E. coli* and 18-3 mm against *B. subtilis*. Biofilm activity observed against *S. aureus* was in the range of 5.1-53.77%. The total phenolic contents (TPC) and total flavonoid (TFC) were in the range of 115.0-173.4 GAE, mg/100 g and 72.23-135.23 CE, mg/100 g respectively. HPLC analysis showed high concentration of sinapic acid and p-coumaric acid. Chemical composition of *H. auriculus* stem was examined by GC-MS analysis. Elemental analysis showed that stem of *H. auriculus* is rich in Calcium.

Keywords: antimicrobial, antioxidant, HPLC, biofilm, cytotoxicity

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Introduction

Plants have been used by human beings as medicine for centuries. There is an urge to enhance knowledge about plants, their biochemistry, effects on human health, their relationship with environment. Thousands of different plants are being used on planet to cure different ailments. There are great number of secondary metabolites including anthocyanins, tannins, flavonoids, carotenoids, alkaloids, saponins and vitamin C etc. These are responsible for antimicrobial and antioxidant activities such as anti-allergic, anti-inflammatory and anti-thrombotic [1-6]. Bacteria have become more drug resistant due to excessive use of antibiotics in livestock, animal feed and aqua culture. The most common infection and drug resistant strains are *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These clinical drug-resistant strains have become difficult to treat and there is a need to search out for safe and reliable antibacterial substances [2, 7-14].

Reactive oxygen species (ROS) in low concentrations are produced in plants during normal metabolic and physiological processes. However, salinity and other drastic conditions result in increased production and accumulation of ROS which are responsible for cell damage and metabolic disorder [15-18]. ROS are liberated due to stress include free radicals ($O_2^{\cdot}$, $O^{-}_{2,}$ ·OH, $ONOO^{-\cdot}$) and non-free radicals (H_2O_2, NO, and ROOH). Eventually, ROS can react with protein, DNA, lipids, causing mutation and damage the cell membrane which results in tissue injuries. Research has revealed that ROS are involved in a number of disorders like Alzheimer, atherosclerosis, inflammation diabetes, cardiovascular and neurodegenerative diseases in human beings [3, 5, 19]. Antioxidants are responsible for certain types of cancer, aging process and offset harmful ROS. They do so by inhibiting oxidative chain reaction, reducing free radical concentration, preventing lipid peroxidation and chelating metal ions. Synthetic antioxidants e.g. Butylated hydroxytoluene (BHT) are considered to be carcinogenic [1].

Among medicinal plants halophytes have gained attention of researcher as they are rich in certain polyphenolic compounds. These are recognized worldwide for treatment of infections to preserve food and in cosmetics. Halophytes are salt tolerant plants which can withstand increased salinity and drought conditions. A large number of halophyte species have been used in traditional medicine [8, 20]. This species is spread in coastal areas of Iran, Turkmenistan, Baluchistan, Tajikistan, Kirghizstan. A very little is known about this specie. So far, no other study of biological activities of *Halothamnus auriculus* (*H. auriculus)* was performed, that's why we propose to investigate *in vitro* antioxidant, antimicrobial, cytotoxicity by hemolysis, biofilm activity, TPC, TFC and mineral composition determination of stem of *H. auriculus* for the first time.

Materials and Methods

The selected plant *H. auriculus* was collected from Quetta, Pakistan. Plant was identified by Botany department, University of Baluchistan, Quetta. The stem of the plant was dried and grounded to fine powder using pestle and mortar. Ground (80 mesh) stem of *H. auriculus* sample (300 g) was subjected to extraction in sequence with six different solvents i.e. *n*-hexane, chloroform, methanol, ethanol, ethyl acetate, *n*-butanol in 300 mL each. The sample was soaked in n-hexane for 7 days with regular shaking for 5 min daily in each solvent. After 7 days solvent containing extracted components was decanted, filtered in beaker and allowed to evaporate under vacuum at 45ºC, using a vacuum rotary evaporator (Ltd., Tokyo, Japan). Residue was re-dissolved in 2 mL of corresponding solvents, transferred to Eppendorf and stored at −4ºC until used for further analysis at room temperature.

The DPPH radical removing activity was performed by taking 1 mL of 0.004% DPPH in methanol mixture poured in 3 mL of plant extracts each. The solutions were kept for 30 min in darkness. Then absorbance of each solution was noted at 517 nm. A low radical scavenging activity results in high absorbance and vice versa [21]. The percentage inhibition of DPPH radical samples was calculated (Eq. 1), where A represents absorbance.

$$\text{DPPH Percent Inhibition} = (A_{Blank} - A_{Sample}/A_{Blank}) \times 100 \quad (1)$$

The TPC in each extract were estimated by Folin-Ciocalteu method as reported by Hussain et al. [22]. The absorbance was measured after 1 h at 765 nm. The calibration curve was designed by assuming absorbance proportional to concentration. TPC in all plant extracts was calculated as gallic acid equivalents (GAE) (Eq. 2).

$$T = C \times V / M \quad (2)$$

...where T presents total phenolics (mg GAE/g plant extract), C represents the concentration of gallic acid from calibration curve, V is the volume of extract in mL and M is the weight of plant extract (g). TFC of plant extracts were studied by following procedure reported by Hussain et al. [22]. The mixture was incubated for 15 min then absorbance of the mixture was measured at 510 nm. The TFC of all six extracts of *H. auriculus* stem were represented as catechin equivalents from the linear reversion curve of catechin. The cytotoxicity assay performed by hemolytic activity against human red blood cells (RBCs) with different extract of stem of *H. auriculus*. The percent Triton X-100 had been taken as the positive and PBS (phosphate buffer saline) as negative control as reported by Kwansa-Bentum et al. [23]. Absorbance was measured on ELISA plate reader at 576 nm.

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$$\% \text{ Hemolysis} = (\text{A of sample} - \text{A of negative control/A of positive control}) \times 100 \quad (3)$$

Antimicrobial activity of each extract was found out by Agar well diffusion method as reported by Oses et al. [24]. To evaluate the biofilm activity of the extracts of *H. auriculus* glass tubes were immunized for the overnight with a loop filled of pure bacteriological culture strain as reported by Bukhari et al. [25]. The mixture was incubated at 37ºC. After two days, the tubes with two percent crystal lavender was rinsed for 7 min and held under distilled water for 5 min. The biofilm was formed on the internal surface of the tube which showed positive result. The nutrient broth was taken as a negative control in the experiment. Concentrations of Zn, Cu, Pb, Ca, Fe and Cr elements in predigested sample of *H. auriculus* stem were determined by AAS (Hitachi Polarized Zeeman AAS, ZZ-8200, Japan). The integral demolition of the plant cell matrix was done as reported by Bukhari et al. [25]. Essential oil of *H. auriculus* stem was extracted by hydro-distillation technique using Clevenger apparatus. 50 g of ground plant sample were dispersed in 400 mL distilled water in 1000 mL round bottom flask and was refluxed for 3 h. Oil was separated from aqueous layer with the help of separating funnel using ethyl acetate followed by evaporation in rotary evaporator leaving behind essential oil. The essential oils were further dried over anhydrous Na_2SO_4, filtered and stored at 4ºC until further analysis.

GC-MS Analysis

The essential oil of *H. auriculus* stem was diluted 200 times with n-hexane for GC-MS analysis. The secondary metabolites present in essential oil of *H. auriculus* stem was identified using TQ7000 series GC-MS equipped with a HP5MS column (30 m × 0.25 mm) and HP 5972 mass spectrometer (Agilent, USA). Helium was used as a carrier gas with flow rate of 1.2 mL min^{-1} and 1µL sample was injected in split mode (1:20). It was programmed temperature run from 50-240ºC at a rate of 5ºC min^{-1} and held at 240ºC for 10 min. The identity of components was confirmed by comparison of their relative retention time and mass spectra with literature [25].

HPLC Analysis

DIONEX Ultimate 3000 HPLC system (Thermo-Fisher, USA) provided with automatic sampler attached to variable UV wavelength detector. The C18 column (Waters, USA) with internal diameter 4.6 mm, height 250 mm and particle size 5 μm was used. It was protected with C18 guard column. The data was analyzed using Chromeleonversion7.2 software [26]. The extraction of the polyphenols was carried out by mixing 50 mL of methanol and 5g plant powder. The mixture was sonicated for 30 min at 30ºC. The methanol extracts were filtered followed by centrifugation at 5000 rpm for 20 min at 25ºC. Methanol was removed in vacuum using a rotary evaporator temperature below 40ºC. (1)Gallic Acid (2) HB Acid (3) Caffeic Acid (4) P -coumaric Acid (5) Sinapic Acid (6) Ferulic Acid were used as standard for HPLC.

Results and Discussion

Amid the halophytes, the genus Halothamnus of the plant family Amaranthaceae was formerly placed in family Chenopodiaceae. Different species of Chenopodiaceae were used for treatment of many diseases. Fever, jaundice, dropsy, liver disease, insecticidal, vascular hypertension were treated by different species of family Amaranthaceae. Eleven secondary metabolites have been isolated from powder of this plant recently [27-32].

The ability of different extracts of stem of *H. auriculus* to donate proton to DPPH free radical and change it into its reduced form DPPH-H (violet to yellow) is accessed in this assay. Antioxidant potential is followed by measurement of absorption maxima between 515-528 nm. DPPH scavenging activity increases as the concentration and degree of hydroxylation of metabolites increases. Table 1 shows the percentage inhibition of DPPH by the extracts. Antioxidant activity of ethanol and n-hexane was significant but was less than standard BHT solution (90.15). *n*-butanol extract showed maximum phenolics (173.4 mg) as compared to methanol (115 mg). No reports were found regarding TPC of *H. auriculus* stem extract by which the results of present work could be compared.

Table 1 represents the amount of TFC of the stem extracts of *H. auriculus.* The maximum amount of TFC was found in ethyl acetate (135.39 mg) and minimum was found in methanol (76.18 mg). The results showed the presence of appreciable amount of TFC which pose the *H. auriculus* stem as excellent source of antioxidants. The percent hemolysis of six extracts of stem of *H. auriculus* was examined to evaluate their toxicity against RBCs. The absolute methanol extract exhibited highest percentage of hemolytic activity (48.4 %) and chloroform extract showed lowest (11.9 %). The positive control (triton X-100) showed 87.4 % and phosphate buffer saline (PBS) used as negative control did not show hemolytic activity. Since hemolysis is even greater than 10% in all cases which reveal that these extracts are toxic to human red blood cells.

All six *H. auriculus* stem extracts showed antimicrobial activity against selected microorganisms. The absolute ethanol extract exhibited greater antimicrobial capability against bacterial strains *E. coli* and *B. subtilis* displaying the maximum inhibition zones 18.0 mm in both cases while *n*-hexane extract produces lowest activity upon both the test strains, and the zone of inhibition against *E. coli* 4 mm and *B.*

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Table 1. Antioxidant activity, TPC and TFC, antimicrobial activity, biofilm activity, hemolysis shown by different extracts of stem of *H. auriculus* and the compounds identified by GC-MS.

Solvent	DPPH inhibition (%)		TPC (GAE mg/100g)	TFC (CE g/100g)
n-hexane	43.82		156.2	84.60
Ethyl acetate	14.84		138.6	135.39
Ethanol	49.45		164.4	72.23
Methanol	34.68		115.0	76.18
Chloroform	17.18		149.8	117.23
n-butanol	41.40		173.4	96.18
BHT	90.15			
	Zone inhibition (mm)		Biofilm Inhibition (%)	Hemolysis RBCs (%)
	E. coli	*B. subtilis*	*S. aureus*	
n-hexane	4	3	50.64	33.49
Ethyl acetate	10	8	51.18	37.69
Ethanol	18	18	17.60	34.66
Methanol	11	10	21.49	48.43
Chloroform	12	4	53.77	11.91
n-butanol	14	8	5.18	22.36
Standard	32	28	78.29	87.40
Peaks	Retention Time		Peak Height	Peak Area
1	43.131		2227831264	2145567662.5
2	43.188		2181382368	2484461297.7
3	43.199		2191782880	1517429867.5
4	43.212		2063460064	2324713357.5
5	43.254		2165313760	2125147616.3
6	43.267		2059896288	635910684.5
7	43.276		2118605280	1139593848.3
8	43.283		2296936672	2139698625.6
9	43299		2446387680	1657321944.0
10	43.313		2410367933.02	2502680765.91

1: Propanoic acid, 2-methyl-, (dodecahydro-6ahydroxy-9a-methyl-3methylene-,9dioxoazuleno[4,5-b] furan-6-yl) methyl ester; 2: 1,7,9-trimethyl-Methanocyclopenta [a]cyclopropa[e]cyclodecen-11one,1a,2,5,5a,6,9,10,10a-octahydro5,5a,6-trihydroxy-1,4 bis(hydroxymethyl); 3: Pregn-4-ene-3,20-dione, 16,17-epoxy; 4: N, N'-Bis (Carbobenzyloxy)-lysine methyl ester; 5: alpha-N-Normethadol 6; 6: Propanoic acid, 2-methyl-, (dodecahydro-6ahydroxy-9a-methyl-3methylene-2,9dioxoazuleno[4,5-b] furan-6-yl) methyl ester; 7: Butanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro5,5a-dihydroxy-4(hydroxymethyl)-1,1,7,9tetramethyl-11-oxo-1H-2,8amethanocyclopenta[a]cyclopropa[e]cyclodecen -6-yl ester; 8: Hydrocortisone Acetate; 9: Betamethasone acetate; 10: Octanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro5,5a-dihydroxy-4(hydroxymethyl)-1,1,7,9tetramethyl-11-oxo1H2,8amethanocyclopenta[a] cyclopropa [e] cyclodecen -6-yl ester

subtilis 3.0 mm respectively. *n*-butanol extract showed marked activity against *E. coli* (14 mm) but low against *B. subtilis* (8 mm) inhibition zone. On the other hand, the standard Ampicillin showed activity 32 mm and 28 mm zone of inhibition against *E. coli* and *B. subtilis* respectively.

Biofilm activity of all these extracts were examined against the *S. aureus*. Chloroform (53 %), ethyl acetate (51.2 %) and n-hexane (50.6 %) extracts showed maximum percent inhibition of biofilm while the standard Ampicillin showed biofilm activity of 78.2 %. *n*-butanol exhibited minimum activity (5.1 %) against

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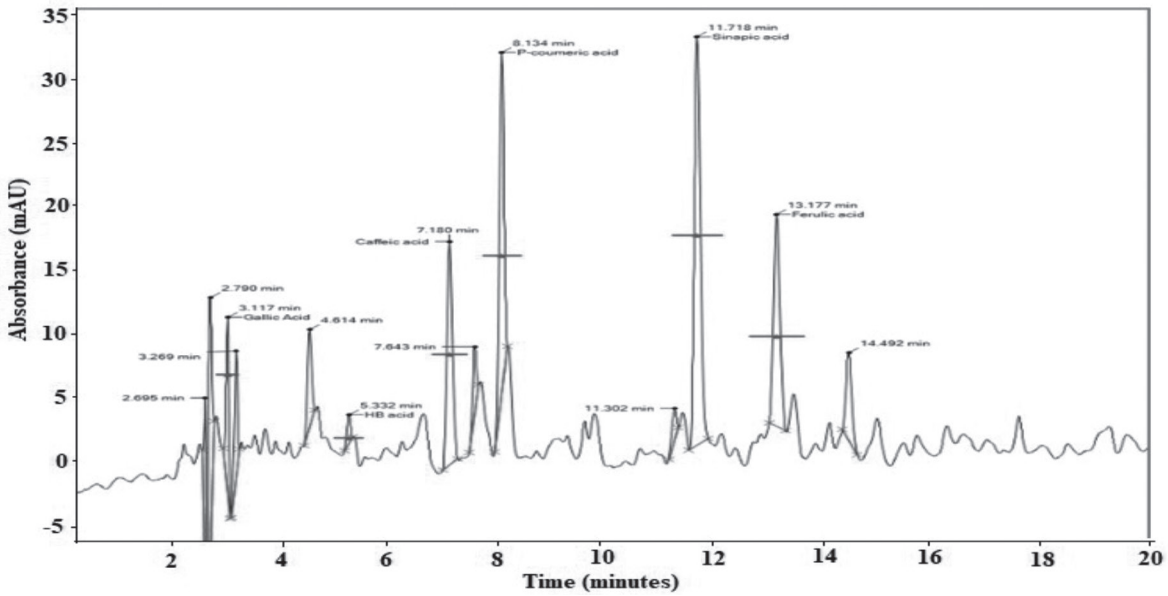

Fig. 1. HPLC chromatogram of methanolic extract of *H. auriculus.*

the *S. aureus.* Results suggest that *H. auriculus* may have high potential against biofilm and may be used as natural source in the pharmaceutical or food industry and seafood preservative. Analysis to determine presence of Ca (2300 ppm), Cr (0.01 ppm), Pb (10 ppm), Cu (0.13 ppm), Zn (31 ppm), in extract of *H. auriculus* stem was performed by AAS. Results showed a great amount of Ca (2300 ppm). This plant could further be investigated to get high amount of Ca and as a source of valuable minerals especially the plants which are used as cattle feed [8, 33]. Fig. 1 shows the HPLC analysis of methanolic extract of *H. auriculus* stem.

Chromatogram shows high concentration of sinapic acid and p-coumaric acid. gallic acid, ferulic acid, HB acid and caffeic acid were also present. However, 13 unidentified phenolic acids showed their presence. Chemical composition of essential oil of *Halothamnus auriculus* stem was determined by GC-MS analysis. The compounds were identified by comparing their relative retention times and mass spectra with literature.

Conclusions

The following conclusions have been drawn:

Total phenolic contents (TPC) were maximum in *n*-butanol (173.4 mg), total flavonoid contents (TFC) were highest in ethyl acetate (135.39 mg). The antioxidant potential of all extracts was assessed by DPPH and the highest percent inhibition (49.45) was shown by ethanol. The chloroform has shown highest inhibition (53%) of biofilm activity. The highest antimicrobial activity was presented by ethanolic extract. HPLC profile also indicate the presence of different phenolics in appreciable amount. High concentration of Ca makes this plant unique. Prominent cytotoxicity against RBCs should further be investigated against tumor cells. This plant could be considered as a potential source of bioactive compounds with beneficial proprieties, suggesting its use in medicine and food industry.

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Conflict of Interest

The authors declare no conflict of interest.

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