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ANTIOXIDANT POTENTIAL OF METHANOL STEM EXTRACT OF *Berberis aristata* DC. AND BERBERINE- A BIOACTIVE COMPOUND ISOLATED FROM *Berberis aristata* DC.

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ABSTRACT

Natural products contain important combinations of ingredients, which may to some extent help to modulate the effects produced by oxidation substrates in biological systems. Oxidative stress has been thought to contribute to the general decline in cellular functions that are associated with many human diseases including Alzheimer disease, amyotrophic lateral sclerosis, Parkinson's disease, atherosclerosis, ischemia/reperfusion neuronal injuries, degenerative disease of the human temporomandibular-joint, cataract formation, macular degeneration, degenerative retinal damage, rheumatoid arthritis, multiple sclerosis, muscular dystry, Inflammatory Bowel Disease, cancers as well as the aging process itself. Increased cellular level of ROS due to oxidative stress can result in an increased steady state level of oxidative DNA damage. The objective of the study was to investigate the antioxidant activity of methanol extract from the stem of *Berberis aristata* DC. and berberine a bioactive isolated compound of *Berberis aristata* DC. by H_2O_2 and DPPH *in-vitro* methods. It was observed that the methanol extract of the stem of *Berberis aristata* DC. and berberine were effectively scavenged free radical activity. Those antioxidant activities were compared to standard antioxidant such as L-Ascorbic acid. Our findings provide evidence that the crude methanol extract of stem of *Berberis aristata* DC. and its bioactive isolated compound (Berberine) is a potential source of natural antioxidant and this justified its uses in folkloric medicines.

KEYWORDS: ***Berberis aristata* DC.*, In vitro* antioxidant, antioxidant activity, Berberine, H_2O_2 and DPPH assay.**

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INTRODUCTION

Free radicals are molecules or parts of molecules caused by the metabolic process of oxygen. Oxygen free radicals (e.g., super oxide, hydroxyl, peroxyl ($RO_2^{\cdot}$), and hydroperoxyl ($HO_2^{\cdot}$) radicals. Nitric oxide and nitrogen dioxide ($^{\cdot}NO_2$) are two free radicals. Peroxynitrite, hypochlorous acid (HOCl), hypobromous acid (HOBr), reactive nitrogen species (RNS), are produced in animals and humans body under physiologic and pathogenic conditions. Free radicals may play an important role in the biological evolution, implicating their beneficial effects on the organisms e.g., Oxygen radicals exert critical actions such as single transduction, gene transcription, and regulation of soluble guanylate cyclase activity in cells[1]. They are hostile and damaging to cells and their functions. They can also cause a chain reaction causing the multiplication of new free radicals. Oxygen free radicals or reactive oxygen species (ROS) the by-product of cell metabolism and are also produced in the body on exposure to sunlight, X-rays, ozone, tobacco smoke, auto-exhaust and other environmental pollutants.[2] The mitochondrial respiratory chain is the major source of super oxide for most cells. Within the mitochondrial matrix, manganese super oxide dismutase converts super oxide to hydrogen peroxide, which can diffuse from mitochondria to the cytoplasm. Increased production of these reactive oxygen species (ROS) by mitochondria is a major contributor to oxidative damage in pathological situations. Complex 1 (NADPH-ubiquinone oxidoreductase) is a major source of superoxide, making it a candidate for increased mitochondrial ROS production and redox signaling. Redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated. Increased levels of ROS directly cause the oxidation of lipids, proteins, and nucleic acids.[3-6] To control the level of ROS and to protect cells under stress condition, plant tissue contain several enzymes and chemical compounds scavenging ROS catalase, peroxidase and a network of low molecular weight antioxidants (ascorbates, glutathione, polyphenols, tocopherols) in addition a whole enzyme array of enzymes is needed for the regeneration of the antioxidants (ascorbate peroxidase, dehydroascorbate, glutathione reductase). Antioxidants found naturally in foods that manage and remove damaging free radicals. The use of antioxidants in food industry is inevitable as they can increase shelf life and prevent oxidation. Synthetic antioxidants such as butylatedhydroxytoluene (BHT), sodium benzoate and butylated hydroxyanisole (BHA) can increase used in food products. However, their use must be controlled due to possible hazards such as carcinogenicity and toxicity. Recent studies indicate that carotenoids, tocopherols, ascorbates and polyphenols are strong natural antioxidants generally found in plants and foods. They are known to act to defuse the volatile toxic molecules of free oxygen radicals. They help to lower the risk of cancer risk, cardiovascular diseases, age related vision disorders, asthma and reduce inflammation.[7] *Berberis aristata* DC. (Family-Berberidaceae) commonly known as “Rashut or Rasanjan (Daruharidra)” / Indian barberry is a spinous, deciduous shrub native in northern Himalaya region is extensively used in various indigenous systems of medicine. The plant is widely distributed from Himalayas to Sri Lanka, Bhutan and hilly area of Nepal. It grows to the height of 1000-3000 meters especially in Kumaon region of Uttara Khand and Chammaba region of Himachal Pradesh. It is also found in the Nilgiri hills in South India.[8-9] It is also used as a blood purifier[10] and as a laxative.[11] The plant is used traditionally in inflammation, wound healing, skin disease, menohrrhagia, diarrhea, jaundice and infection of eyes. Pharmacological studies on the plant reveal the proven activity of it as a hypoglycemic, antimalarial, antibacterial, antifungal, antipyretic, anti-inflammatory, hepatoprotective, antioxidant and anticancer agent. Phytochemical studies show that plant *Berberis aristata* DC. contains alkaloid berberine, oxyberberine, berbamine, aromoline, a protoberberine alkaloid karachine, palmatine, oxycanthine, taxilamine, tannins, sugar and starch.[12]

MATERIALS AND METHODS

Stem of *Berberis aristata* DC. was collected from the forest area of Mussoorie, Dehradun, India in the month of February 2008. The herbarium of *Berberis aristata* DC. was prepared and submitted to National Bureau of Plant and Genetic Resources (NBPGR) for authentication. The specimen was authenticated by botanist Dr. K.C. Bhatt, Senior Scientist, National Bureau of Plant and Genetic Resources (NBPGR), Pusa Campus , New Delhi. Authentication reference number is NHCP/NBPGR/2008/4.

Figure1
***Berberis aristata* DC.**

***Extraction of stem of Berberis aristata* DC.**
The stem of *Berberis aristata* DC. was washed with water after dead cell scraping, chopped into smaller pieces for air-drying at room temperature for 7 days and coarsely powdered. 200 g of pulverized plant material was extracted by soxhlet extraction with 500 mL of methanol for 48 hours. The obtained extract was filtered and evaporated to dryness using a rotary evaporator (R-114, Buchi, Switzerland) at reduced temperature and pressure. The solvents and other chemicals used were of the analytical grade.

Preliminary Phytochemical Screening
Phytochemical investigation were performed by doing different qulalitative chemical tests including tests for alkaloids, glycosides, tannins, carbohydrates, saponins, proteins and amino acids, resins, lipids/fats, phenolic compounds, flavonoids in methanol extract of stem of *Berberis aristata* DC.[13-14]

Experimental Methodology
The antioxidant activity of stem extract of *Berberis aristata* DC. (BAME) and its bioactive isolated compound were determined by two different *in vitro* methods such as, DPPH free radical scavenging assay and H_2O_2 free radical scavenging methods. The extract and the isolated compound berberine were dissolved in distilled water and methanol respectively. All the assays were carried out in triplicate and average value were recorded.

Chemicals
2,2-Diphenyl-1- picryl-hydrazyl (DPPH), Hydrogen Peroxide (H_2O_2), L-Ascorbic acid were obtained from Sigma Aldrich Co., St. Louis, USA and was used as a free radical scavenger and standard drug respectively. All other chemicals/solvent used were of analytical grade.

Determination of H_2O_2 Free Radical Scavenging Activity
The ability of BAME and its isolated compound berberine in scavenging H_2O_2 was determined. A solution of H_2O_2 (40mM) was prepared in phosphate buffer saline (PBS-pH 7.4). H_2O_2 concentration was determined spectrophotometrically, at 230nm (Shimadzu UV-Vis 1700) measuring absorption. The extract at different concentration ranging from 25-500µg/ml were added to H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2.[15] The percentage of H_2O_2 scavenging of both the extract and standard compound were calculated as Follows

$$H_2O_2 \text{ Scavenged (\%)} = \frac{(A_{cont} - A_{test})}{(A_{cont})} \times 100$$

Where,
A_{cont} = Absorbance of control reaction
A_{test} = Absorbance of test reaction

Determination of 2, 2-Diphenyl-1-Picryl Hydrazyl (DPPH) Free Radical Scavenging Activity

The free radical scavenging activity of BAME, isolated compound berberine and L-Ascorbic acid were measured in terms of hydrogen donating ability or radical scavenging ability by means of the stable radical DPPH (2, 2-diphenyl-1-picryl hydrazyl). 0.1 mM solution of DPPH in ethanol was prepared and 1.0 mL of this solution was added to 3.0 ml of the extract solution in water at different concentrations 25- 500 µg/ml and isolated compound berberine 10-50 µg/ml in ethanol. After 30 min. incubation at room temperature in the dark environment the absorbance were measured at 517 nm by UV-Visible spectrophotometer (Shimadzu UV-Vis 1700). A purple to yellow colour change was observed. Methanol was used as a blank. The measurements were performed in triplicate and the results were averaged. Lower absorbance values of the reaction mixture indicate higher free radical scavenging activity.[16] The capability to scavenge the DPPH free radical was calculated using the following equation

$$\text{DPPH Scavenged (\%)} = \frac{(A_{cont} - A_{test})}{(A_{cont})} \times 100$$

Where,

A_{cont} = Absorbance of control reaction

A_{test} = Absorbance of test reaction

Statistical Analysis

Experimental results were expressed as mean ± SD in triplicate measurement and IC_{50} was graphically determined.

Isolation of the bioactive compound from the BAME

Healthy stems were collected from the *in vivo* plants, dried at shade and powdered mechanically. 100 g of the powdered stem was extracted with methanol in a Soxhlet apparatus for 48 h. The extract was concentrated under reduced pressure using a rotary flash evaporator (Büchi, Switzerland); 10 g of extract was separately acidified with 2% HCl. The filtered acidic solution was extracted with diethyl ether (Et_2O) to remove neutral materials and made basic (pH 8-9) with sodium bicarbonate (Na_2CO_3) and extracted with dichloro methane (CH_2Cl_2). The CH_2Cl_2 solution was concentrated and dried in the desiccator. The crystals of isolated compound thus obtained was powdered and stored in an airtight amber coloured bottle. The crude extract and isolated compound were tested qualitatively for the presence of alkaloids and chromatographed by TLC method using the solvents n- butanol: glacial acetic acid: water in the ratio of 12:3:4 v/v/v. Then R_f values were calculated for extract and isolated compound. The characterization of isolated compound was carried out by HPLC, FTIR, ^{1}HNMR, ^{13}CNMR and MASS spectral studies. Waters HPLC was used with 3µl injection and 10 minutes run time. The FTIR spectrum was recorded with KBr pelletes on a Perkin-Elmer 1710 FTIR spectrophotometer. The ^{1}HNMR spectra were obtained by Bruker AMX (400 MHZ) Spectrophotometer. DMSO and $CDCl_3$ were used as solvent respectively in ^{1}HNMR and ^{13}CNMR. The mass spectrum was recorded on a Bruker micro TOF-Q II 10262 ESI Spectrophotometer.

RESULTS AND DISCUSSION

The % yield of BAME obtained was to be 12.8%w/w on dry basis, appeareance of BAME was brownish yellow coloured dried powder. The preliminary phytochemical analysis of BAME revealed the presence of alkaloid, glycosides, tannins, phenolic compound and flavanoids.

Table 1
Preliminary phytochemical studies of methanol extract of stem of Berberis aristata DC. (BAME)

S. No.	Plant constituents	Methanol Extract
1	Alkaloid	+++
2	Carbohydrate	++
3	Glycoside	+
4	Saponin	-
5	Phenolic compound	++
6	Flavonoid	++
7	Phytosterol	+
8	Triterpenoid	-
9	Tannin	++

+++ Significantly Present, ++ Moderately Present, + Slightly Present, - Absent

TLC Study of BAME

The presence of phytoconstituents was further confirmed by thin layer chromatography and their R_f values have been presented. TLC profiling of BAME gives an impressive result that directing towards the presence of number of phytochemicals. In the present study, TLC profiling of BAME in n-butanol: ethyl acetate: acetic acid: water (3:5:1:1) solvent system indicated the presence of 4 different types of phytochemicals. From the thin layer chromatography studies revealed that the R_f values obtained from the methanol extract can be interpreted as 4 different compounds may be present with R_f values 0.85, 0.74, 0.53, 0.48.

In Vitro Antioxidant Activity By H_2O_2 Free Radical Scavenging Activity

Table 2
H_2O_2 free radical scavenging activity of BAME

Dose (µg/ml)	% Inhibition	IC_{50}
BAME 25	21.20 ± 1.626	98 µg/ml
BAME 50	40.66 ± 1.308	
BAME 100	50.86 ± 0.7487	
BAME 200	60.54 ± 2.421	
BAME 500	71.86 ± 1.527	
L-Ascorbic Acid	87.07 ± 1.302	

Values are expressed in mean ± SD, n = 3, BAME represent the methanol extract of the stem of Berberis aristata DC.

DPPH (2, 2-Diphenyl-1-Picryl Hydrazyl) Free Radical Scavenging Activity

Table 3
DPPH free radical scavenging activity of BAME

Dose (µg/ml)	% Inhibition	IC_{50}
BAME 25	22.60 ± 1.714	122 µg/ml
BAME 50	37.91 ± 1.354	
BAME 100	46.90 ± 1.589	
BAME 200	58.22 ± 0.9260	
BAME 500	72.84 ± 1.599	
L-Ascorbic Acid	87.11 ± 3.781	

Values are expressed in mean ± SD, n = 3, BAME represent the methanol extract of the stem of Berberis aristata DC.

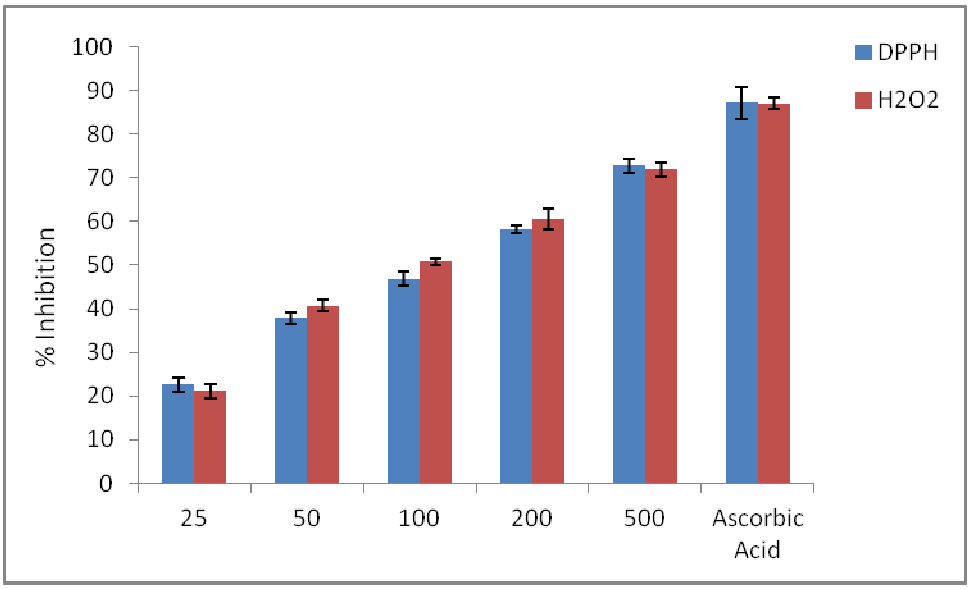


Figure 2
Percentage Inhibition of BAME in H_2O_2 and DPPH models

Isolation of bioactive molecule from the BAME

The isolated compound was obtained as a yellowish needle shaped crystals.

Figure 3
Crystals of isolated compound from BAME

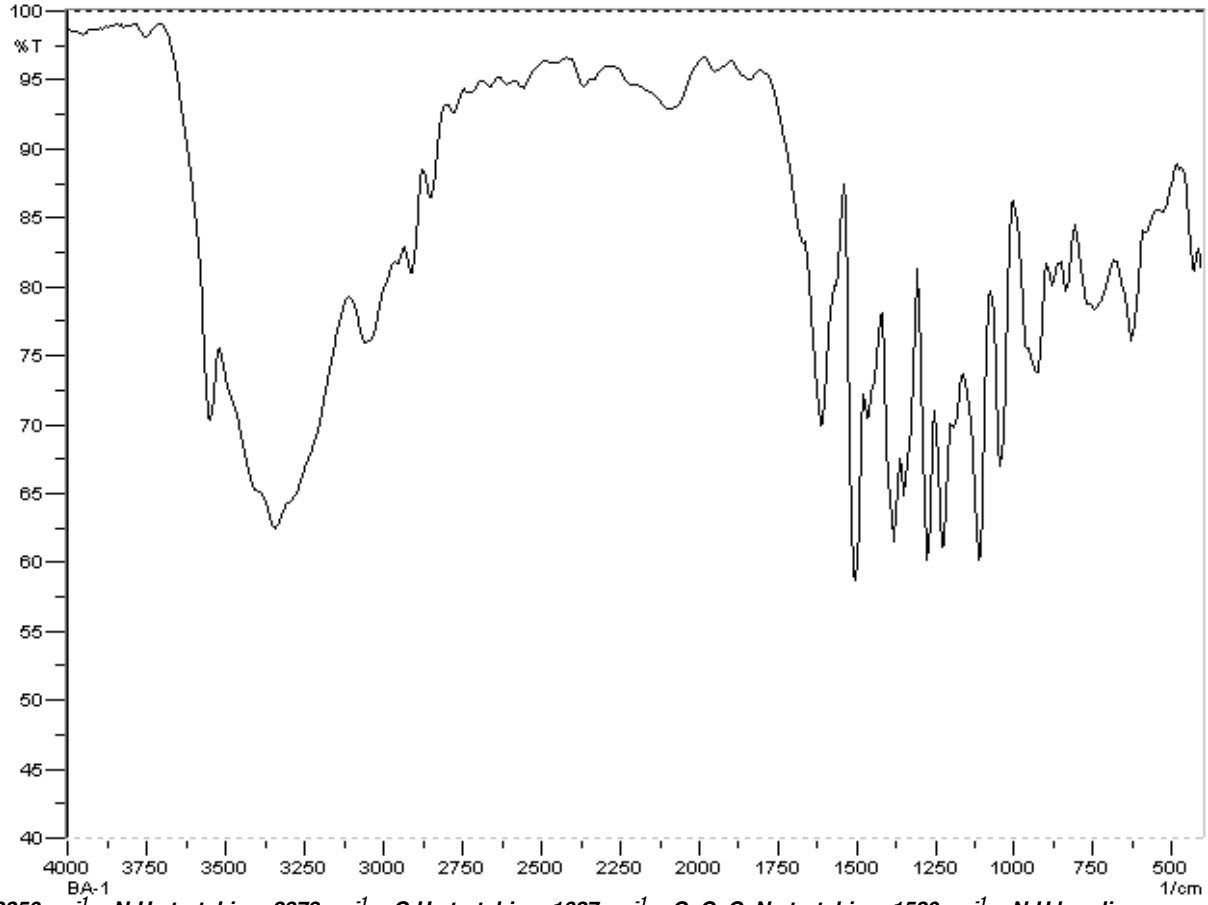
TLC study of isolated bioactive compound from BAME

Table 4
Percentage yield, solvent system and R_f value of isolated compound from BAME

Nameof extract	% Yield	Solvent system	R_f Value	Inference
Isolated Compound	2.8% w/w from stem of *Berberis aristata* on dry wt. basis	n-Butanol: Glacial acetic acid: Water (12:3:4)	0.55	A single R_f value indicates a single compound may be isolated

Spectral Studies

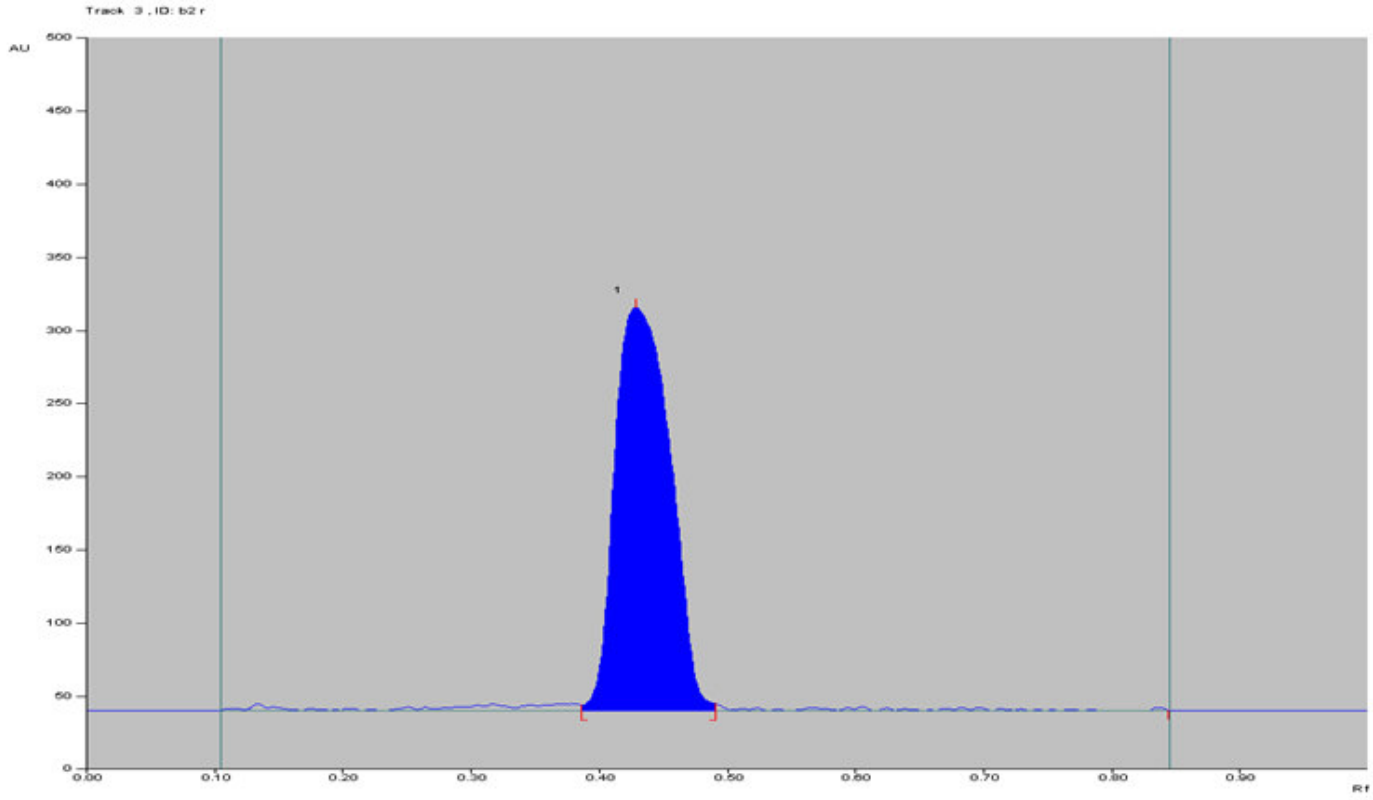
FTIR Finger print analysis of isolated compound from BAME



3350 cm^{-1} = N-H stretching, 2878 cm^{-1} = C-H stretching, 1627 cm^{-1} = C=C, C=N stretching, 1520 cm^{-1} = N-H bending, 1055 cm^{-1} =C-O stretching.

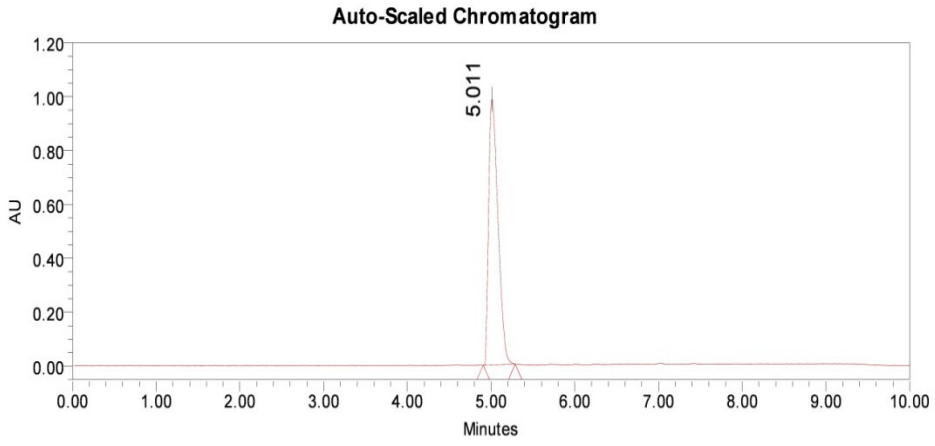
HPTLC Finger print analysis of isolated compound from BAME

Mobile Phase used was n-propanol: acetic acid: water (8: 1: 1 v/v/v), the isolated compound was solublized in ethanol, Saturation time was 20 minutes.



Peak	Start Position	Start Height	Max Position	Max Height	Max%	End Position	End Height	Area	Area%
1	0.39 Rf	5.4 AU	0.43 Rf	386.2 AU	100.00 %	0.49 Rf	6.8 AU	13516.9 AU	100.00 %

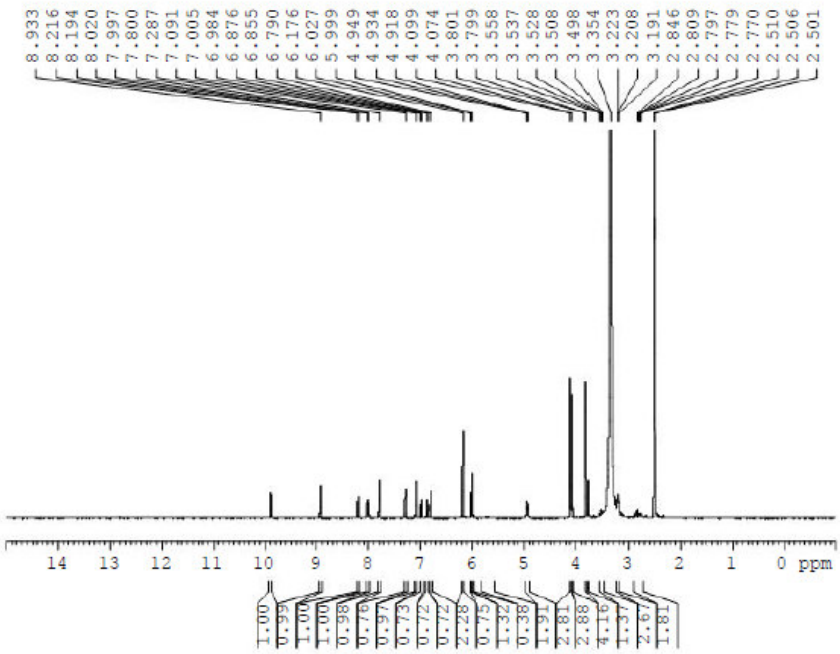
HPLC chromatogram of isolated compound from BAME



Peak Results

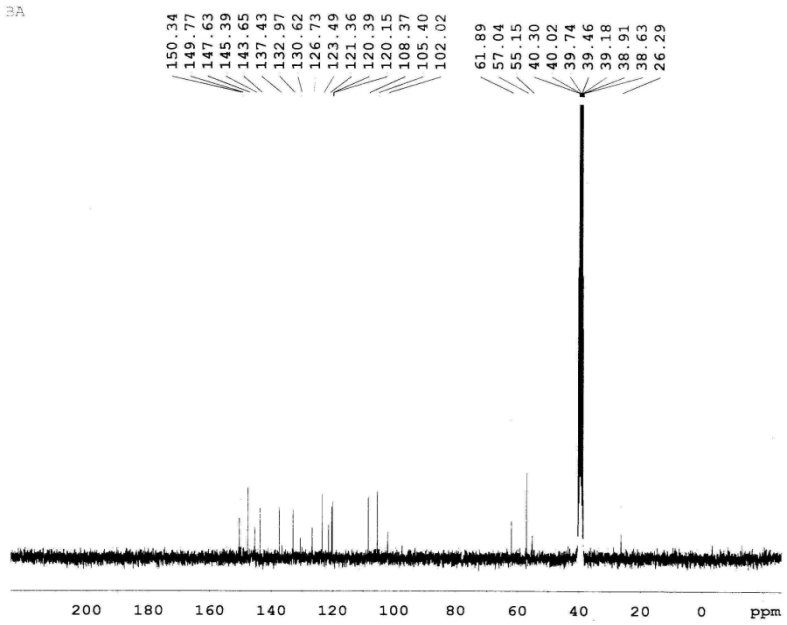
RT	Area	Height	% Area
5.011	7655353	987158	100

^{1}H NMR peak of isolated bioactive compound from BAME



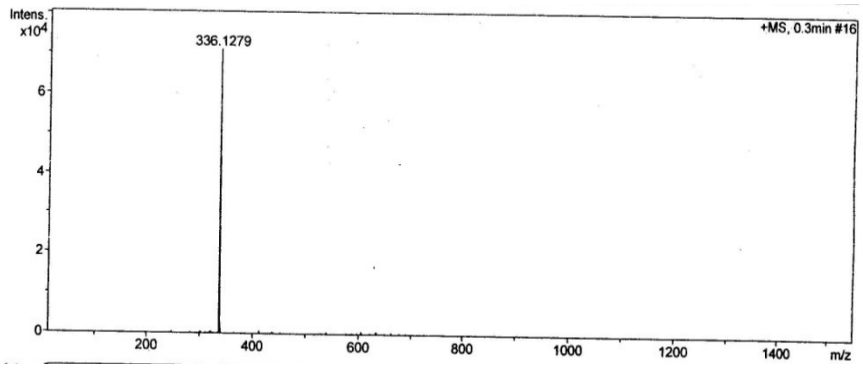
^{1}H NMR (DMSO-d_6): δ 8.19 (^{1}H, d, J=9.2 Hz, H-11), 7.99 (^{1}H, d, J=9.2 Hz, H-12), 7.80 (^{1}H, s, H-1), 7.09 (^{1}H, s, H-8), 6.79 (^{1}H, s, H-4), 6.17 (2H, brs, O-CH_2-O), 5.99 (^{1}H, s, H-13), 4.09 (3H, brs, OMe), 4.07 (3H, brs, OMe), 3.80 (2H, dd, J=1.6, 2.0 Hz, H_2-6), 2.50 (2H, dd, J=1.6, 2.0 Hz, H_2-5).

^{13}CNMR peak of isolated bioactive compound from BAME

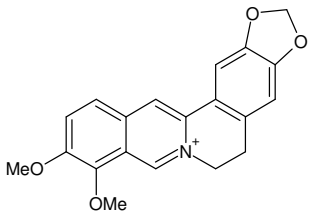


^{13}C NMR (DMSO-d_6): δ 105.40 (C-1), 147.63 (C-2), 149.77 (C-3), 108.37 (C-4), 130.62 (C-4a), 26.29 (C-5), 55.15 (C-6), 145.39 (C-8), 120.15 (C-8a), 143.65 (C-9), 150.34 (C-10), 126.73 (C-11), 123.49 (C-12), 132.97 (C-12a), 120.39 (C-13), 137.43 (C-13a), 121.36 (C-13b), 102.02 (O-CH2-O), 61.89 (OMe), 57.04 (OMe).

MASS spectrum of isolated bioactive compound from BAME



On the basis of phytochemical screening the isolated bioactive compound was found as an isoquinolone alkaloid. The isolated compound afforded molecular peak [M+1] 336 in ESI mass spectrum suggesting molecular formula $C_{20}H_{18}NO_4$. The experimental values of IR and MASS were used to predict the molecular structure, atomic stretching, possible molecular functional group, etc., for the confirmation of berberine alkaloid present in the BAME. Combining all the data we could arrive to the conclusion that the isolated compound is Berberine with a molecular formula $C_{20}H_{18}NO_4$.



BERBERINE
IUPAC NAME
9,10-dimethoxy-5,6-dihydro [1,3]dioxolo [4,5-*g*] isoquino[3,2-*a*] isoquinolin-7-ium

In Vitro Antioxidant Assay of Berberine By H_2O_2 Free Radical Scavenging Activity

Dose (µg/ml)	% Inhibition	IC_{50}
Isolated Compound 10	35.41 ± 1.530	31 µg/ml
Isolated Compound 25	45.15 ± 1.140	
Isolated Compound 50	77.98 ± 2.464	

Values are expressed in mean ± SD, n = 3, isolated compound, Berberine obtained from the BAME

termination of 2, 2-Diphenyl-1-Picryl Hydrazyl (DPPH) Free Radical Scavenging Activity

Dose (µg/ml)	% Inhibition	IC_{50}
Isolated Compound 10	32.72 ± 0.8277	32 µg/ml
Isolated Compound 25	41.33 ± 1.006	
Isolated Compound 50	80.42 ± 1.430	

Values are expressed in mean ± SD, n = 3, Isolated Compound Berberine obtained from the BAME

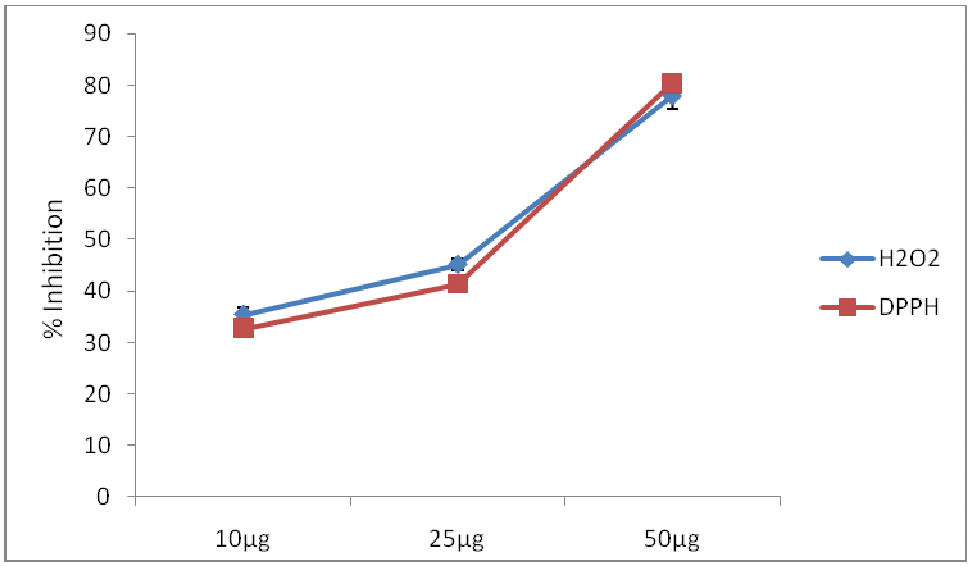


Figure 5
Percentage Inhibition of Berberine in H_2O_2 and DPPH models

DISCUSSION

Free radical scavenging activity depends on the change of absorbance by different drug sample in different concentrations. The change in absorbance are checked by UV-Vis. spectrophotometer at λ_{max} 235 and 517 nm in H_2O_2 and DPPH free radical scavenging method respectively. After calculating the % inhibition of free radical scavenging activity it had seen that more potent drug produced more % inhibition. In this study BAME extract showed % inhibition in both H_2O_2 and DPPH free radical scavenging activity in a dose dependent manner. The standard drug, L-Ascorbic acid showed 87.07% and 87.11 %inhibition of H_2O_2 and DPPH free radical scavenging activity respectively, whereas BAME 500µg/ml showed 71.86 and 72.84 %inhibition. The IC_{50} was graphically calculated and were found to be 98µg/ml and 122 µg/ml in H_2O_2 and DPPH free radical scavenging activity respectively. This result showed BAME has strong antioxidant potential in a increasing order of BAME 25 <BAME 50< BAME 100 <BAME 200 <BAME 500. The isolated berberine (highest dose i.e. 50µg/ml) from the BAME shown 77.98% and 80.42% scavenging activity respectively. IC_{50} was found as 31µg/ml and 32µg/ml respectively by H_2O_2 and DPPH free radical scavenging activity.

CONCLUSION

Searching plant sources may bring new natural products into pharmaceutical, cosmetic and food production. Based on the results of the present study, BAME and its bioactive isolated compound Berberine showed highest antioxidant activity at a dose of 50µg/ml. The high antioxidant capacity observed for BAME and Berberine suggesting that it may play a vital role in preventing human diseases in which free radicals are involved, such as cancer, ageing and cardiovascular diseases.

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