IN-VITRO ANTIOXIDANT ACTIVITY OF ALCOHOLIC AND AQUEOUS EXTRACTS OF ACHYRANTHES ASPERA (ASCLEPIADACEAE)

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Summary

Free radicals are implicated for more than 80 diseases including diabetes mellitus, arthritis, cancer, liver diseases, ageing, conditions in which oxidative stress is prominent. In treatment of these diseases, antioxidant therapy has gained an utmost importance. In Indian system of medicine *Achyranthes aspera* is an important medicinal plant and its leaf, seed paste or root juice has been used in various ailments and as health tonic. To understand the mechanisms of pharmacological actions, the in-vitro antioxidant activity of alcoholic and aqueous extracts of leaf, root and seeds of *Achyranthes aspera* was investigated for activity of DPPH free radical, superoxide anion radicals and nitric oxide radical scavenging assay spectrophotometrically. Total phenolic contents in all extracts were determined by Folin Ciocalteu reagent. The highest free radial scavenging activity was found with aqueous extract of seeds and methanolic extract of root of *Achyranthes aspera*. IC_{50} values of aqueous extracts of seeds were found 147.3 ± 3.74, 138.45 ± 0.921, 155.06 ± 0.29 mcg ml^{-1} in DPPH free radical, superoxide anion radical and nitric oxide radical assay. The total phenolic contents of aqueous extract of seeds of *Achyranthes aspera* were found 529.9. From the results, we infer that antioxidant property may be related to the antioxidant polyphenolic contents present in the aqueous extract of seeds of *Achyranthes aspera*. These results clearly indicate that *Achyranthes aspera* is effective against free radical mediated diseases.

Keywords: *Achyranthes aspera*, Free radicals, Scavenging ability, Polyphenols.

Introduction

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals (1). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (2). The most common reactive oxygen species (ROS) include superoxide ($0_2^{\cdot}$) anion, hydrogen peroxide (H_20_2), peroxyl (ROO-) radicals, and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (3). In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin.

Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers (4, 5). Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments activities, no side effects and economic viability (6). Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic. etc. (7). They were also suggested to be a potential iron chelator (8, 9). Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. In Indian system of medicine *Achyranthes aspera* is an important medicinal plant and its leaf, seed and root paste or root juice has been used in various ailments and as health tonic (10-13). Achyranthes aspera Linn., belonging to family Amaranthaceae, is commonly found as a weed on way side and at waste places throughout India. It is known as Apamarg in Sanskrit, Aghedo and Aghedi in Gujarati, Chirchira and Chirchitta in Hindi and Prickly chaff flower in English. It is widely used for asthmatic cough, snakebite, hydrophobia, urinary calculi, rabies, influenza, piles, bronchitis, diarrhea, renal dropsy, gonorrhea and abdominal pain (14, 15, 16, 17, 18). A powder of dried leaf mixed with honey is useful in the early stages of asthma (19). One of the drugs from Siddha system of medicine, Naayuruvi kuzhi thailum has A. aspera as the primary constituent is reported to be quite effective in the management of asthma (20). *Achyranthes aspera* contains mainly flavonoids, alkaloids (21), saponins (22), steroids and terpenoides (23). Therefore, the objectives of the present study were to investigate the in vitro antioxidant activity of methanolic and aqueous extracts of root, seed and leaf of *Achyranthes aspera* through the free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide scavenging assay.

Methods

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio- barbituric acid (TBA) were purchased from S. D Fine Chemical Ltd. India and Hi Media Comp. Pvt. Ltd. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials:

Roots, leaves and seeds of *Achyranthes aspera* were procured from the surrounding fields of Mehsana in the month of October-November. They were identified by Dr. Ritesh Vaidya, Department of Botany, Mehsana Urban Bank Institute of Bioscience, Ganpat University, Mehsana. The roots, leaves and seeds of the plant were dried in shade at room temperature. The dried roots, leaves and seeds were subjected to size reduction to a coarse powder with the help of Wiley's mill.

A. Preparation of methanolic extract (MLAA, MSAA, MRAA):

The powder was packed in a soxhlet apparatus and extracted with 95% methanol for 18 hrs. Appearance of colourless solvent in the siphon tube was taken as the termination of extraction. The extract was then transferred into the previously weighed empty beaker and evaporated to a thick paste on the water bath, maintained at 50^{o}C to get methanolic extract. The marc was finally air dried thoroughly to remove all traces of the solvent. The percentage yield was calculated.

B. Preparation of aqueous extract (AQLAA, AQSAA, AQRAA):

About 100g of powder was taken in a round bottom flask (2000ml) and macerated with 500 ml of distilled water and 10 ml of chloroform (preservative) for seven days with shaking for every eight hour in a closed vessel. Then the marc was removed by filtering the extract, and then it was concentrated on a water bath at 50^{o}C to get a semi solid mass.

These six extracts were stored in an airtight container in a refrigerator below 10^{o}C. The two extracts were examined for their colour and consistency.

Total Phenolic content

Total phenolic content was determined using Folin - Ciocalteau method (17-19). Each of the 100µl of samples of MLAA, MSAA, MRAA, AQLAA, AQSAA and AQRAA was taken in to 25ml volumetric flask, to which 10ml of water and 1.5ml of Folin Ciocalteau reagent were added. The mixture was then kept for 5 min. and to it 4ml of 20% w/v sodium carbonate solution was added the volume was made up to 25ml with double distilled water. The mixture was kept for 30 minute until blue color develops. The samples were then observed at 765 nm in UV- visible spectrometer Shimadzu, UV-1601, Japan. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure. The DPPH free radical scavenging activity, Super oxide free radical scavenging activity and Nitric oxide scavenging activity were calculated using the following formula:

$$\% \text{ Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance} \times 100}{\text{Control absorbance}}$$

DPPH free radical scavenging activity (20, 21)

4.3mg of DPPH (1, 1-Diphenyl –2- picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solution was added to 3ml methanol and absorbance was taken immediately at 516nm for control reading. Different volumes of samples of MLAA, MSAA, MRAA, AQLAA, AQSAA and AQRAA were diluted with methanol up to 3ml and to each 150 µl DPPH was added. Absorbance was taken after 15 min. at 516nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC50 value for each extracts was calculated.

Super Oxide free radical scavenging activity (22, 23)

100µl Riboflavin solution [20 µg], 200µl EDTA solution [12 mM], 200µl methanol and 100µl NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and

reaction mixture was diluted up to 3ml with phosphate buffer [50mM]. The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5min.This is taken as control. Different volumes, of samples of MLAA, MSAA, MRAA, AQLAA, AQSAA and AQRAA were taken and diluted up to 100 µl with methanol, to each of this, 100µl Riboflavin, 200µl EDTA, 200 µl methanol and 100µl NBT was mixed in test tubes and further diluted up to 3ml with phosphate buffer. Absorbance was measured after illumination for 5min. at 590nm on UV visible spectrometer Shimadzu, UV-1601, Japan. IC50 value for each extracts was calculated.

Nitric Oxide scavenging activity (24-26)

Different concentrations of MLAA, MSAA, MRAA, AQLAA, AQSAA and AQRAA (50-600 µg/ml) were taken in separate tubes and the volume was uniformly made up with methanol to each tube 2.0 ml, of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. Ascorbic acid was used as positive control. IC50 value for each extracts was calculated.

Results

The test for total phenolic content was carried out on individual extract and standard drug. It was observed that AQRAA and ASRAA had highest phenolic content then rest of the extracts as showed in Table-1. In the DPPH Free radical scavenging activity, the AQSAA and MRAA showed very potent scavenging activity then rest of the extracts, while the standard drug (Ascorbic acid) showed highly potent scavenging activity as mentioned in Table-2. The half inhibition concentration (IC50) of AQSAA was 147.3 ± 3.74.

Table- 1 Total phenolic content

Substance	Total Phenolic content in µg/ml
1. MLAA	154.4 ± 4.00
2. MSAA	220.4 ± 3.00
3. MRAA	367.9 ± 5.50
4. AQLAA	172.9 ± 4.50
5. AQSAA	529.9 ± 2.50
6. AQRAA	274.9 ± 5.50

* The value is expressed as µg of Gallic acid equivalent / ml of sample
* The regression values and correlation of regression of Gallic acid were $y = 0.001x + 0.0264$ and $R^2 = 0.9982$

Table-2 Free radical scavenging activity against DPPH

Dose	IC_{50} in µg/ml	Regression equation	R^2
Ascorbic acid	11.70 ± 0.010	y = -0.0312x + 0.7336	0.9955
MLAA	460.97 ± 8.47	y = -0.0008x + 0.7511	0.9977
MSAA	370.92 ± 3.97	y = -0.001x + 0.7498	0.9942
MRAA	258.78 ± 2.35	y = -0.0014x + 0.7312	0.9985
AQLAA	372.7 ± 3.70	y = -0.001x + 0.7528	0.9935
AQSAA	147.3 ± 3.74	y = -0.0024x + 0.725	0.9938
AQRAA	325.7 ± 11.03	y = -0.0011x + 0.7408	0.9960

The sign R^2 is correlation of regression

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (27). The superoxide anion radical scavenging activity of the extract from *Achyranthes aspera* assayed is shown in Table-3. The superoxide scavenging activity of *Achyranthes aspera* was increased markedly with the increase of concentrations. The half inhibition concentration (IC50) of AQSAA and MRAA were 138.45 ± 0.92 and 211.18 ± 1.87 µg/ml. These results suggested that *Achyranthes aspera* had important superoxide radical scavenging effect.

Table-3 SO free radical scavenging activity

Dose	IC_{50} in µg/ml	Regression equation	R^2
Ascorbic acid	12.79 ± 0.66	y = -0.0288x + 0.7746	0.9964
MLAA	334.54 ± 1.42	y = -0.0012x + 0.8063	0.9956
MSAA	303.88 ± 2.80	y = -0.0013x + 0.7828	0.9957
MRAA	211.18 ± 1.87	y = -0.0019x + 0.7954	0.9963
AQLAA	349.33 ± 6.75	y = -0.0011x + 0.7834	0.9970
AQSAA	138.45 ± 0.92	y = -0.0028x + 0.7702	0.9948
AQRAA	332.5 ± 2.83	y = -0.0012x + 0.7912	0.9976

The sign R^2 is correlation of regression

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (28). *Achyranthes aspera* extracts moderately inhibited nitric oxide in dose dependent manner (Table-4). The half inhibition concentration (IC_{50}) of AQSAA and MRAA were 155.06 ± 0.29 and 189.04 ± 0.68 µg/ml. These results suggested that *Achyranthes aspera* had important nitric oxide radical scavenging activity.

Table-4 NO free radical scavenging activity

Dose	IC_{50} in µg/ml	Regression equation	R^2
Ascorbic acid	14.04± 0.80	y = -0.028x + 0.831	0.9969
MLAA	518.62 ± 3.50	y = -0.0008x + 0.8242	0.9974
MSAA	419.92 ± 1.07	y = -0.001x + 0.8377	0.9972
MRAA	189.04 ± 0.68	y = -0.0022x + 0.8288	0.9963
AQLAA	417.32 ± 2.42	y = -0.001x + 0.8395	0.9963
AQSAA	155.06 ± 0.29	y = -0.0026x + 0.8078	0.9946
AQRAA	349.58 ± 1.50	y = -0.0012x + 0.8426	0.9948

The sign R^2 is correlation of regression

Discussion

The phytochemical analysis of *Achyranthes aspera* extracts contain rich source of polyphenol. Polyphenol used for the prevention and cure of various diseases which is mainly associated with free radicals (29).

Antioxidants are known to protect the body against free radial mediated toxicities. A large numbers of plants have shown potent antioxidant activity (30, 31). The present study was undertaken to test for successive extracts of *Achyranthes aspera* leaves, seeds and roots for in-vitro antioxidant activity using DPPH, Super oxide and Nitric oxide methods. The crude aqueous extracts of seeds and methanolic extract of roots exhibited potent anti-oxidant activity with low IC_{50} values in these three methods. However the activity was found to be less than the standard used. These extracts (AQRAA & MSAA) could scavenge super oxide and nitric oxide radicals.

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichometrically depending on the number of electrons taken up (32). From the results it may be postulated that both the plant extracts have hydrogen donors thus scavenging the free radical DPPH.

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, alzheimer's and arthritis
(33). Oxygen reacts with the excess NO to generate nitrite and peroxy nitrite anions, which act as free radicals. In the present study the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25^0 c was reduced by AQSAA and MRAA. This may be due to the antioxidant principles in the extracts, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. This could explain its meagre antioxidant effect in this method.

Demonstration of the antioxidant potential of this herb, especially in view of the presence of a rich spectrum of bio active molecules of therapeutic significance, makes them likely candidates for bio activity guided fractionation of useful phytomolecules.

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