IN-VITRO ANTIOXIDANT ACTIVITY OF VARIOUS FRACTIONS OF ACHYRANTHES ASPERA (ASCLEPIADACEAE)

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Summary

Apamarg (*Achyranthes aspera* L., Asclepiadaceae) is an erect or procumbent, annual or perennial herb found throughout in India and other tropical countries. 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. Despite its importance, few reports exist in the literature regarding the chemistry or antioxidant activity of this species. In present study, the methanolic and aqueous extracts of roots and seeds of *Achyranthes aspera* was fractionated using five solvents of different polarity, viz., hexane, chloroform, ethyl acetate, methanolic and water. For each fraction, the total phenolic content was estimated as in vitro antioxidant activity assessed using the 1,1-diphenyl-2-picrylhydrazyl, superoxide anion radicals and nitric oxide radical scavenging assays spectrophotometrically. The fractions demonstrated different degrees of potency within each assay; however, the observed pattern was not necessarily replicated between assays indicating the importance of the use of more than one screening technique to estimate the antioxidant activity of plant fractions. This study demonstrates antioxidant activity of *Achyranthes aspera.*

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

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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 (14-18). A powder of dried leaf mixed with honey is useful in the early stages of asthma (19). *Achyranthes aspera* contains mainly alkaloids, saponins, steroids and terpenoides (20).
Methanolic and aqueous extracts of roots and seeds of *Achyranthes aspera* showed potent antioxidant activity in all three assays and also both extracts containing highest total phenolic content (21). Therefore, both extracts chose for fractionation with solvents in non polar to polar order. The in-vitro antioxidant potency of all fractions was assessed with three in-vitro assays.

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 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 and seeds of the plant were dried in shade at room temperature. The dried roots and seeds were subjected to size reduction to a coarse powder with the help of Wiley's mill.

Preparation of Fractions:

Air-dried alcohol and aqueous extracts of seed and root of plant were packed into a paper thimble and inserted into a Soxhlet apparatus. These extracts were exhaustive fractionated sequentially with 100 ml hexane, chloroform, ethyl acetate, methanol and water. These all fractions were stored in an airtight container in a refrigerator below 10°C. The fractions were dissolved in appropriate solvents prior to analysis depending upon solubility. The nomenclature of fractions was given like HFSAA, HFRAA (Hexane Fraction of Seed and Root of *A. aspera),* CFSAA, CFRAA (Chloroform Fraction of Seed and Root of *A. aspera),* EFSAA, EFRAA (Ethanolic Fraction of Seed and Root of *A. aspera),* MFSAA, MFRAA (Methanolic Fraction of Seed and Root of *A. aspera),* WFSAA, WFRAA (Water Fraction of Seed and Root of *A. aspera)* and used same in text.

Total Phenolic content

Total phenolic content was determined using Folin - Ciocalteau method (22-24). Each of the 100µl of samples of HFSAA, CFSAA, EFSAA, MFSAA, WFSAA, HFRAA, CFRAA, EFRAA, MFRAA and WFRAA was taken in to 25 ml volumetric flask, to which 10 ml of water and 1.5 ml of Folin Ciocalteau reagent were added. The mixture was then kept for 5 min. and to it 4 ml of 20% w/v sodium carbonate solution was added the volume was made up to 25 ml 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:

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

DPPH free radical scavenging activity (23, 25)

4.3 mg 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 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different volumes of samples of HFSAA, CFSAA, EFSAA, MFSAA, WFSAA, HFRAA, CFRAA, EFRAA, MFRAA and WFRAA were diluted with methanol up to 3 ml and to each 150 µl DPPH was added. Absorbance was taken after 15 min. at 516 nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value for each fraction was calculated.

Super Oxide free radical scavenging activity (26, 27)
100 µl Riboflavin solution (20 µg), 200 µl EDTA solution (12 mM), 200µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution (0.1mg) were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer (50 mM). The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 min. This is taken as control. Different volumes, of samples of HFSAA, CFSAA, EFSAA, MFSAA, WFSAA, HFRAA, CFRAA, EFRAA, MFRAA and WFRAA were taken and diluted up to 100 µl with methanol, to each of this, 100 µl Riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT was mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value for each fraction was calculated.

Nitric Oxide scavenging activity (28-30)
Different concentrations of HFSAA, CFSAA, EFSAA, MFSAA, WFSAA, HFRAA, CFRAA, EFRAA, MFRAA and WFRAA (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. IC_{50} value for each fraction was calculated.

Results

Total Phenolic Content:
The test for total phenolic content was carried out on individual fraction and standard drug. It was observed that WFSAA and MFRAA had highest phenolic content then rest of the fractions as showed in Table-1.

Table- 1 Total phenolic content

Fractions	**Total Phenolic content in µg/ml**
1. HFSAA	208.9 ± 2.5
2. CFSAA	230.9 ± 7.5
3. EFSAA	159.9 ± 2.5
4. MFSAA	142.4 ± 3.0
5. WFSAA	460.4 ± 3.02
6. HFRAA	174.9 ± 4.5
7. CFRAA	95.9 ± 5.5
8. EFRAA	123.9 ± 3.5
9. MFRAA	327.9 ± 5.5
10. WFRAA	186.9 ± 3.5

* The value is expressed as µg of Gallic acid equivalent / ml of sample
* The regression values and correlation of regression of Gallic acid were $y = 0.001x + 0.0256$ and $R^2 = 0.9992$

DPPH Free radical scavenging activity:
In the DPPH free radical scavenging activity, the WFSAA and MFRAA showed very potent scavenging activity then rest of the fractions, while the standard drug (Ascorbic acid) showed highly potent scavenging activity as mentioned in Table-2. The half inhibition concentration (IC_{50}) of WFSAA and MFRAA were 96.38 ± 2.15 and 143.68 ± 1.87.

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 (31). The superoxide anion radical scavenging activity of the fractions from *Achyranthes aspera* assayed is shown in Table-2. The superoxide scavenging activity of *Achyranthes aspera* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of WFSAA and MFRAA were 101.08 ± 1.77 and 159.28 ± 4.87 µg/ml. These results suggest that *Achyranthes aspera* had important superoxide radical scavenging effect.

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 (32). *Achyranthes aspera* fractions' moderately inhibited nitric oxide in dose dependent manner (Table-2). The half inhibition concentration (IC_{50}) of WFSAA and MFRAA were 123.44 ± 1.93 and 208.90 ± 5.14 µg/ml. These results suggest that *Achyranthes aspera* had important nitric oxide radical scavenging activity.

Table- 2 In-vitro antioxidant activity of different fractions of AQSAA and MRAA against DPPH, SO, NO assays

Fractions	Inhibitory Concentration (IC_{50} µg/ml)		
	DPPH	SO	NO
1. Ascorbic acid	14.10 ±0.69	13.68 ± 0.10	13.81 ± 0.65
2. HFSAA	583.66 ± 16.91	621.85 ±3.71	540.28 ± 7.78
3. CFSAA	633.96 ±6.18	721.33 ±4.75	614.53 ±8.25
4. EFSAA	408.9 ± 22.4	461.30 ±2.13	480.44 ± 5.16
5. MFSAA	385.84 ±3.98	424.02 ± 2.87	431.75 ±4.50
6. WFSAA	96.38 ± 2.15	101.08 ±1.77	123.44 ±1.93
7. HFRAA	418.62 ±6.70	469.33 ± 5.78	462.58 ±6.03
8. CFRAA	310.63 ±5.70	324.69 ± 4.15	340.95 ± 5.45
9. EFRAA	377.62 ± 4.87	421.72 ±5.77	412.75 ±8.00
10. MFRAA	143.68 ±1.87	159.28 ± 4.87	208.90 ± 5.14
11. WFRAA	464.92 ± 8.32	490.58 ± 5.25	489.14 ±17.36

Discussion

The phytochemical analysis of *Achyranthes aspera* fractions contain rich source of polyphenol. Polyphenol used for the prevention and cure of various diseases which is mainly associated with free radicals (33).
Antioxidants are known to protect the body against free radial mediated toxicities. A large numbers of plants have shown potent antioxidant activity (34, 35). The present study was undertaken to test for successive fractions of *Achyranthes aspera* seed and root for in-vitro antioxidant activity using DPPH, Super oxide and Nitric oxide methods. The water fraction of seed and methanolic fraction of root 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 fractions (WFSAA & MFRAA) 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 (36). From the results it may be postulated that both the fractions 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 (37). 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 WFSAA and MFRAA. This may be due to the antioxidant principles in the fractions, 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 isolation of useful phytomolecules.

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