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In vitro Antioxidant Activity of Aqueous and Alcoholic Extracts of *Andrographis paniculata* Whole Plant Powder by ABTS free Radical Scavenging Assay

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ABSTRACT: The present investigation aimed to evaluate the antioxidant activities of whole plant extracts of *Andrographis paniculata* (APWP) by an *in vitro* model ABTS free radical scavenging assay. Gallic acid, at a concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 µg/ml was used as a reference inhibitor in the ABTS assay, which showed 15.88, 32.25, 45.62, 61.91 and 72.39 per cent inhibition of ABTS free radicals, respectively, with IC_{50} value of 1.5 µg/ml (1.33 - 1.70 µg/ml). Both ethanolic and aqueous extracts of APWP at a concentration of 5, 10, 25, 50 and 100 µg/ml showed 2.46, 5.36, 12.64, 24.52 and 41.85 per cent inhibition and 3.68, 6.51, 17.54, 33.27, and 57.30 per cent inhibition of ABTS free radicals, respectively. The IC_{50} value of the ethanolic and aqueous extract samples in the ABTS radical scavenging assay was 150.2 µg/ml (105.72 - 261.30 µg/ml) and 87.75 µg/ml (67.87 - 117.62 µg/ml), respectively.
So, the *in vitro* studies clearly showed that the whole plant extracts of *A. paniculata* have a significant antioxidant activity and the aqueous extract was found to be more effective in the hydroxyl radical scavenging activity with a better IC_{50} value than the ethanolic extract of APWP. It could therefore be concluded that the free radical scavenging activity of the whole plant extract of *Andrographis paniculata* might be responsible for the therapeutic properties.

Keywords: *Andrographis paniculata*, antioxidant activity, ABTS, free radical scavenging assay.

INTRODUCTION

Free radicals are generally very reactive molecules possessing an unpaired electron, which are produced continuously in cells either as by-products of metabolism or by leakage from mitochondrial respiration (Slater, 1984). The free radicals produced *in vivo* include the active oxygen species such as superoxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Oxygen free radicals are responsible for many pathological conditions (De Zwart *et al*., 1999). Free radicals and Reactive Oxygen Species (ROS) cause DNA damage, lipid peroxidation and protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders such as cancer, cardiovascular diseases, inflammatory diseases, asthma and aging (Vani *et al*., 1997; Jadhav and Bhutani 2002). Free radicals like the hydroxyl radical, hydrogen peroxide, super-oxide anion, etc. mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Super-oxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorphonuclear leucocytes to the endothelium and stimulation of platelet aggregation (Aragon *et al*., 1998).

Plants and their products have abundant phytochemicals and have been proven to be good sources of potential antioxidants (Harman, 1992). Today consumers prefer natural antioxidants for use as nutraceuticals, biopharmaceuticals and as food additives (Rohman *et al*., 2010). As these naturally obtained antioxidants have fewer or no side effects, they are preferred over synthetically derived antioxidants, which have genotoxic effects. Therefore, investigations of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous.

Andrographis paniculata (Burm. f.) Nees, a small annual herb, member of the family Acanthaceae, is found in Sri Lanka, Pakistan, Java, Malyasia, Indonesia and throughout India. In India, it is cultivated in Uttar Pradesh, Himachal Pradesh, Assam, Madhya Pradesh, Tamil Nadu, Karnataka and Kerala. In Tamil Nadu, it is cultivated in Thanjavur, Salem, Erode, Vilupuram, Tiruchengode and Palayamkottai (Elumalai *et al.*, 2016). It is commonly known as Nila Vembu or

Siriyanangai (Tamil), Kalmegh (Hindi) or King of bitters (English).

A. paniculata has been prominently used in at least 26 Ayurvedic formulations as confirmed from the Indian Pharmacopoeia; It has been widely used in Chinese medicine as an anti-inflammatory and antipyretic drug for the treatment of cold, fever and laryngitis (Deng *et al.*, 1982). The plant is also one of the components of Nilavembu Kudineer Chooranam, a polyherbal Siddha preparation containing an equal proportion of nine plants, which is successful in the prevention and treatment of chikungunya, dengue viral fever, and COVID-19 in humans and hence approved for use by the Government of India (Kavinilavan *et al.*, 2017). The plant has been reported to have several secondary metabolites having anti-oxidant properties with a wide range of therapeutic applications.

Hence, the present study was formulated to study the *in vitro* antioxidant activity of alcoholic and aqueous extracts of *A. paniculata* whole plant powder by an *in vitro* method, ABTS free radical scavenging assay. Recent research (Yadav and Singh 2023; Zhang *et al*., 2023) has further underscored the role of plant-based antioxidants in mitigating oxidative stress and associated chronic disorders. However, there is still a lack of comparative studies on the antioxidant activity of whole-plant aqueous and alcoholic extracts of *Andrographis paniculata* using the ABTS assay with updated post-2020 methodologies. This research addresses that gap.

MATERIALS AND METHODS

Collection of whole plants of *Andrographis paniculata.* *Andrographis paniculata* whole plants of around 120 days old were randomly collected as per the procedures of Jain (2016) from Herbal Garden of Department of Pharmacology and Toxicology, Veterinary College and Research Institute, Namakkal and District Forest Office (DFO), Mohanur Road, Namakkal (Situated at an average elevation of 218 metres above mean sea level with latitude of 11.23°North and longitude of 78.17° East), Tamil Nadu, India.

Authentication of the plant. The collected whole plants of *A. paniculata* were authenticated for their family and species by the Botanical Survey of India, Southern Regional Centre, Tamil Nadu Agricultural University Campus, Coimbatore, Tamil Nadu, India.

Preparation of *A. paniculata* whole plant powder (APWP). The collected whole plants and roots were washed under running tap water, spread on wetting papers and dried in shade for four weeks with frequent tilting. The whole plants were chaffed and further shade dried for another week, followed by mechanical grinding. Then the whole plants were pulverised and sieved to get a fine powder (Rajat Chakraborty and Tilottama Dey 2016). The whole plant powder was stored in air-tight containers for extract preparations.

Preparation of extracts of *A. paniculata* whole plant powder. Aqueous and alcoholic extracts of six samples of *A. paniculata* whole plant powder were prepared by adding 20 grams of dry powder to 200 ml of distilled water and 70 percent ethanol, respectively, that was kept in a rotary shaker for 48 hours, filtered through Whatman No. 1 filter paper and then incubated at 50˚C for 48 hours to evaporate the solvents. The dried extract was collected (Malahubban *et al*., 2013; Amin Mir *et al*., 2016) and stored in an airtight container for further investigations.

ABTS free radical scavenging assay

Principle. The ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption that can be followed spectroscopically. The relatively stable ABTS radical has a green colour and is measured at 734nm (Gulcin *et al*., 2010).

Materials required

For ABTS Assay

- ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] (A1888, Sigma, USA, store at RT OR 11557, Fluka, USA, store at RT).
- Positive control: Gallic acid [3,4,5-trihydroxy benzoic acid] (G7384, Sigma, USA, store at RT).
- Other reagents:
  - ♦Phosphate Buffer Saline (P3813, Sigma, USA, store at RT),
  - ♦Ammonium persulphate (A0550, Rankem, India, store at RT).
- Microwell plate: 96 well flat, clear plate, Cat. no. 980040, Tarsons.

Preparation of working solutions

For ABTS Assay

- **Phosphate buffer saline 10mM [PBS] (pH-7.4 at 25°C):** 9.9 g (one sachet) of PBS is dissolved in 1000ml of de-ionized water.
- **Ammonium persulfate [APS] (2.45mM):** 5.59mg of APS is dissolved and made up to 10ml of 10mM PBS pH 7.4.
- **Positive control:**

Stock 1: 2.5mg of Gallic acid is dissolved & made up to 10 ml 10mM PBS pH 7.4 (250µg/ml). Further dilutions are made as required.

- **ABTS (7mM):**38.41mg of ABTS is dissolved in 10ml of 10mM PBS pH 7.4.
- **ABTS radical solution:**
  - ♦**Stock 1 (3.5mM ABTS):**10ml of 7mM ABTS + 10ml of 2.45mM APS is mixed & incubated in the dark (stored in an amber coloured bottle) for 16hrs at 25°C to produce the ABTS radicals.
  - ♦**Working solution (0.238mM ABTS):** 680µl of Stock 1 is made up to 10ml with 10mM PBS pH 7.4. The working solution should have an absorbance of ≥1.0 at 734nm.

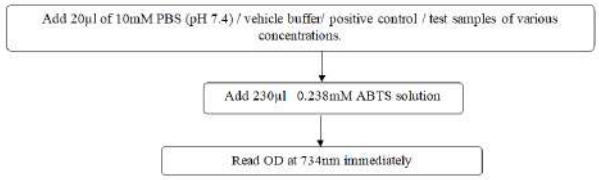
Final assay concentration. In a 250 µl reaction volume, the final concentrations are 0.219mM of ABTS and 10mM of PBS (pH 7.4).

Sample preparation. 6.25 mg of sample was dissolved in 500µl of methanol and the volume was made up to 5ml with 10mM PBS, pH 7.4. Further dilutions were made as required.

Procedure. ABTS assay was carried out as per the method of Gulcin *et al*. (2010). In brief, a 250µl total reaction volume contains 20µl of PBS/vehicle

buffer/positive control/various concentrations of test solution and 230µl 0.238mM ABTS solution. The reaction mixture was incubated at 25°C for 30 minutes, following which the absorbance was read at 734nm using a micro-well plate reader (Molecular devices Versamax microplate reader).

Assay flow diagram



RESULTS AND DISCUSSION

ABTS free radical scavenging assay. The results of *in-vitro* evaluation of anti-oxidant activities of aqueous and ethanolic extracts of APWP by ABTS free radical scavenging assay are presented in Table 1 and the 96-well plate used for this study is depicted in Plate 1.

In this study, ethanolic and aqueous extracts of APWP were evaluated for its possible ABTS free radical scavenging activity. Gallic acid, at a concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 µg/ml, was used as a reference inhibitor, which showed 15.88, 32.25, 45.62, 61.91 and 72.39 percent inhibition of ABTS free radicals, respectively with IC_{50} value of 1.5 µg/ml with a range of 1.33 to 1.70 µg/ml.

Both ethanolic and aqueous extracts of APWP were tested for its ABTS free radical scavenging activity at a concentration of 5, 10, 25, 50 and 100 µg/ml which showed 2.46, 5.36, 12.64, 24.52 and 41.85 per cent inhibition and 3.68, 6.51, 17.54, 33.27, and 57.30 per cent inhibition of ABTS free radicals for ethanolic and aqueous extracts, respectively.

The IC_{50} value of the ethanolic and aqueous extract samples in the ABTS radical scavenging assay was found to be 150.2 µg/ml with a range of 105.72-261.30 µg/ml and 87.75µg/ml with a range of 67.87-117.62µg/ml, respectively. The aqueous APWP extract exhibited a greater antioxidant activity with a better IC_{50} value than the ethanolic APWP extract.

The antioxidant properties demonstrated by this study confirmed the previous findings of several researchers (Lin *et al*., 2009; Prakash *et al*., 2011; Sharma and Joshi 2011; Adegboyega and Oyewole 2015; Premanath and Nanjaiah 2015; Low *et al*., 2015; Sivakumar and Rajeshkumar 2015; Sinha and Raghuwanshi 2020) These findings are consistent with recent studies (Yadav and Singh 2023; Zhang *et al*., 2023) highlighting the impact of solvent polarity on phytochemical extraction efficiency and antioxidant potential.

The results of this study corroborated with Sharma and Joshi (2011), who concluded that the alcoholic extract of AP leaves exhibited appreciable free radical scavenging activity as compared to the aqueous extract.

In harmony with the findings of the present study, Prakash *et al*. (2011) reported that the leaf extract of AP is more effective in the hydroxyl radical scavenging activity and showed that the AP leaf extract has a significant antioxidant activity.

Aqueous extract of APWP showed better antioxidant activity, which is primarily attributed to a higher amount of polyphenolic phytochemicals present in aqueous extracts. Water-soluble tannins, high molecular weight phenolic compounds, not only primary antioxidant donating hydrogen atom or electron, but also function as secondary antioxidants. The major pharmacological activity of *A. paniculata* resides in the diterpene compound (Andrographolide), which was shown to exhibit better antioxidant activity than ethanolic and aqueous extracts. Despite the presence of all these active principles, the antioxidant activity was less than the standard antioxidant (Gallic acid) tested, which could be improved by adopting better extraction procedures.

Table 1: *In vitro* antioxidant activity of ethanolic and aqueous extracts of APWP by ABTS free radical scavenging assay.

Sample	Conc. (µg/ml)	%Inhibition	IC_{50} (µg/ml) (95% C.I.)
Gallic Acid (Reference Inhibitor)	0.5	15.88	**1.5 (1.33-1.70)**
	1.0	32.25	
	1.5	45.62	
	2.0	61.91	
	2.5	72.39	
Ethanol extract	5	2.46	**150.2 (105.72-261.30)**
	10	5.36	
	25	12.64	
	50	24.52	
	100	41.85	
Aqueous extract	5	3.68	**87.75 (67.87-117.62)**
	10	6.51	
	25	17.54	
	50	33.27	
	100	57.30	

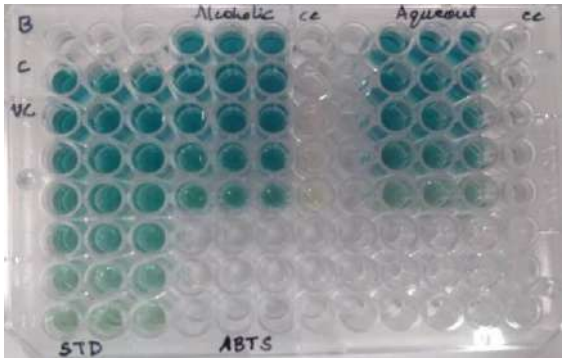


PLATE 1 : ABTS assay - 96 well plate.

CONCLUSIONS

The results of the present study clearly showed that both ethanolic and aqueous extracts of APWP have potent anti-oxidant properties, which can be used as natural antioxidants for treatment of wide variety of disease conditions ranging from pyrexia to COVID-19 not only in humans, but also in animals.

FUTURE SCOPE

Advanced extraction methods, such as ultrasound-assisted or supercritical fluid extraction, could be explored to enhance the yield of active compounds. In vivo validations and formulation development for nutraceutical and pharmaceutical applications are also recommended.

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