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Assay of Biological Antioxidant Activity of Ashwagandha Fruits by Hydrogen Peroxide Radical Scavenging Activity Method

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Abstract
In India indigenous remedies have been used in treatments of various diseases.The Ashwagandha (*Withania somnifera*) enjoys a holy position in Ayurveda-an Indian indigenous system of medicine. Hence, in the current study we aimed for assessment of *in-vitro* anti-oxidant activity of Ashwagandha fruit by hydrogen peroxide radical scavenging activity method.Fruits of Ashwagandhawassubjected to successive solvent extraction by continuous hot extraction (Soxhlet) with methanol and double distilled water. The total phenolic content (TPC) estimation in methanolic and aqueous (aq.) fruit extracts of Ashwagandha was carried out using Folin-Ciocalteu method. The antioxidant assay was carried out by *in-vitro* model using hydrogen peroxide (H_2O_2) radical scavenging activity method. Results revealed that TPC of methanolic fruit extract of Ashwagandha was found to be highest (49.45 GAE, mg/100g) as compared to TPC of aq. fruit extract of Ashwagandha (32.68 GAE, mg/100g). At 200 µg/mL concentration, methanolic and aq. fruit extract of Ashwagandha showed H_2O_2 radical scavenging activity of 152.40% and 112.07% respectively. These findings depicted that methanolic fruit extract *of* Ashwagandhapossess superior antioxidant activity than aqueous fruit extract of Ashwagandha. Hence it could be concluded that methanolic fruit extract of Ashwagandha could be employed for the management of oxidative stress conditions and could be considered for development of natural antioxidant drugs.
Keywords: Ashwagandha, Fruits, Methanolic extract, Hydrogen peroxide scavenging, Antioxidant

Introduction
Ashwagandha (*Withania somnifera*) is a small, woody shrub in the Solanaceae family that grows about two feet in height. It can be found growing in Africa, the Mediterranean and India. An erect, evergreen, tomentose shrub, 30-150 cm high, found throughout the drier parts of India in waste places and on bunds. Roots are stout fleshy, whitish brown; leaves simple ovate, glabrous, those in the floral region smaller and opposite; flowers inconspicuous, greenish or lurid-yellow, in axillary, umbellate cymes; berries small, globose, orange-red when mature, enclosed in the persistent calyx; seeds yellow, reniform. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring.[1]

Figure 1: Showing Ashwagandha plant and fruits

Ashwagandha is widely distributed in the drier parts of tropical and sub-tropical zones, ranging from the Canary Islands, The Mediterranean region and Northern Africa to Southwest Asia including Israel, Jordan, Egypt, Sudan, Iran, Afganistan, Baluchistan, Pakistan and India. In India the plant grows wild in North Western regions extending to mountainous regions of Punjab, Himachal Pradesh and Jammu up to an altitude of 1500 m. It grows successfully in sandy loam or light red soils. A soil pH range of 7.5 to 8 is ideal. It is cultivated in an area of about 5000 hectares in India mainly in drier parts of Rajasthan, Madhya Pradesh,Andhra Pradesh and Uttar Pradesh.[2]

Oxidative Stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or organ, caused by the reactive oxygen species. Reactive oxygen species *viz.*superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxy nitrite radicals play an important role related to the pathogenesis of various important diseases.[3,4] Antioxidants refer as a broad range of compounds which have ability to neutralize free radicals by donating one of their electrons. It acts in different ways by preventing the propagation of the oxidative chain reaction by scavenging free radicals by being part of redox antioxidant network, or by regulating gene expression.[5] It is believed that natural antioxidants rich food can actually lower risks of degenerative diseases and prevent oxidative stress therefore recently many medicinal plants have been studied for their antioxidant properties.[6.7]

In India indigenous remedies have been used in treatments of various diseases such as neurologic degeneration, diabetes etc... Ashwagandha is used as rejuvenator, anti-ageing and adaptogenic agent. With these viewpoints the present study was conducted with the main objective of evaluation of *in-vitro*anti-oxidant activity of Ashwagandha fruits by hydrogen peroxide radical scavenging activity method.

Materials and Methods

Collection of Ashwagandha Fruits

The fruits of Ashwagandhawere purchased from the local marketsof Chikkaballapura, Karnataka, India. The locally purchased fruits of Ashwagandha were sprayed with ethanol, and then shade dried at room temperature for 24-48 hrs. The dried fruits of Ashwagandha were crushed to fine powder with help of electric grinder and stored in airtight containers for further analysis.

Extraction

Approximately 100 g of dried and finely powdered fruits of *Ashwagandha* were subjected to successive solvent extraction by continuous hot extraction (Soxhlet) with 500 mL of methanol and double distilled water. The extracts were concentrated by distilling the solvent in a rotary flash evaporator. The extracts were preserved in airtight containers and stored at room temperature until further use.[8,9]

Estimation of Total Phenolic Content (TPC)

The TPC was determined by the modified method of Folin-Ciocalteu method as described by Hatami et al.[10] Briefly, 500 µL of different concentrations of extracts was mixed with 0.5 mL of 10-fold diluted Folin-Ciocalteu reagent. After 5 min 0.5 ml of 7.5% Sodium carbonate solution, 4.5 ml of double distilled water were added, vortexed and incubated in a dark place for 120 min, the optical density was measured at 760 nm against a blank. The TPC was calculated on the basis of the calibration curve of gallic acid standards (10 ppm-100 ppm) and expressed as gallic acid equivalents (GAE), in milligrams per 100 grams of the sample (mg/100g sample).

***In-vitro* Antioxidant Activity Assay**

Hydrogen peroxide (H_2O_2) radical scavenging activity method

The hydrogen peroxide radical scavenging activity of methanolic fruit extracts of Ashwagandha was determined using modified method as described by Nabhavi et al.[11]Briefly, a solution of hydrogen peroxide (2 mmol/L) was prepared in phosphate buffer (pH 7.4). Five different concentrations of methanolic and aqueous (aq.) fruit extracts of Ashwagandha and standard Ascorbic acid *viz.* 25, 50, 100, 150, and 200 µg/ml in double distilled water in H_2O_2 solution to final volume of 2 mlincubated for 10 mins against a blank solution containing phosphate buffer (pH 7.4) without H_2O_2. Absorbance was recorded at 230 nm against a blank solution. The percentage inhibition of H_2O_2 scavenging activity of methanolic and aq. extracts of Ashwagandha fruits was calculated using the following formula;

$\%\ \text{Scavenged}\ (H_2O_2) = [(A_o - A_1)/A_o] \times 100$

Where,

A_o - Absorbance of the control

A_1 - Absorbance in the presence of the sample of extract and standard

Results and Discussion

Total Phenolic Content (TPC)

The results of TPC of methanolic and aq. fruits extract of Ashwagandha was represented in Table 1 and plotted in Figure 2. Results revealed that TPC of methanolic fruit extract of Ashwagandha was found to be highest (49.45 GAE, mg/100g) as compared to TPC of aq. fruit extract of Ashwagandha (32.68 GAE, mg/100g).

Table 1: Total phenolic content (TPC) Ashwagandha fruit extracts

Solvents	**Total Phenolic Content (TPC, GAE mg/100g)**
Methanol	49.45 ± 1.56
Water	32.68 ± 0.98

Values were expressed Mean ± S.D; n=3; GAE, Gallic acid equivalents

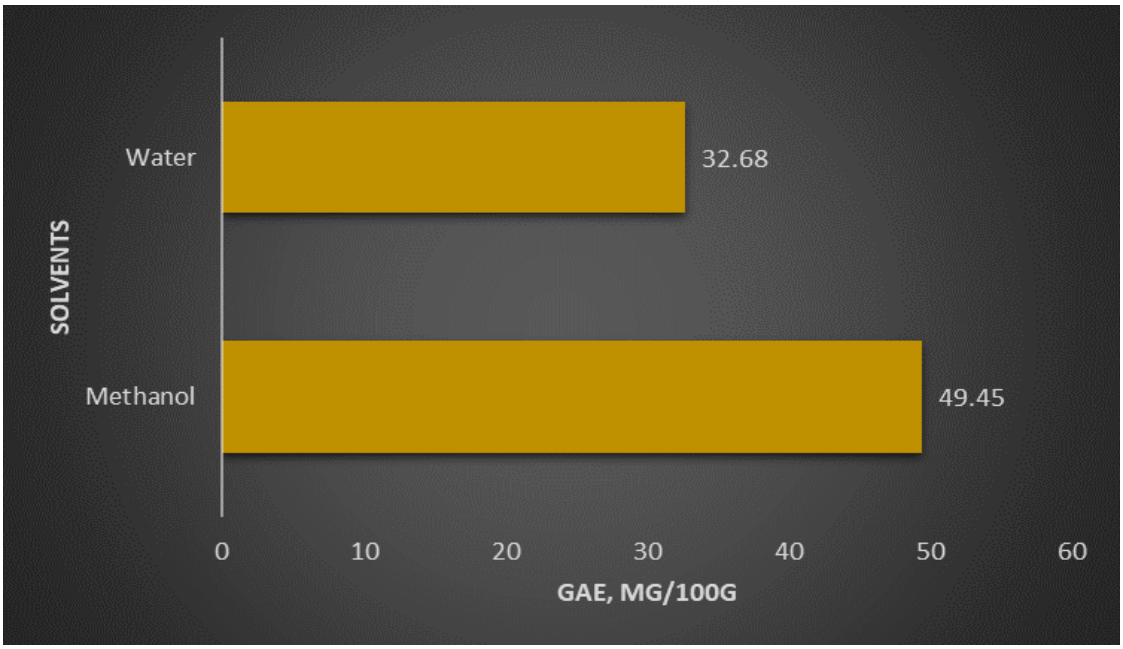

Figure 2: Total phenolic content (TPC) of Ashwagandha fruit extracts

It was revealed in the literature that methanol is the best solvent for catechin extraction, whereas a better yield for procyanidins was obtained with 70% acetone. Oki et al., detected a value of this variable 3 times higher when extracts of red-hulled rice were obtained using methanol rather than water.[12] Arts et al., reported that both acetone and methanolgive similar maximum catechin yields, but the extraction was influenced by concentration and type of the solvent, which affect the yield of catechins.[13] Yet methanol was not as good as ethanol in obtaining total soluble solids but it showed great extractability as compared to ethanol and water. Variations were justified by the well-known tendency of phenols to combine themselves through polymerization reactions; due to the more significant area of charge delocalization, oligomers exerted a higher antiradical activity than the original monomers.[14,15]

Determination *In-vitro* Antioxidant Activity Assay

Hydrogen peroxide (H_2O_2) radical scavenging activity method

The methanol and aq. fruitextracts of Ashwagandhashowed a dose dependent H_2O_2 radical scavenging activity. The H_2O_2 radical scavenging activity in methanol extract was comparatively superior to aq. fruit extract of Ashwagandha. At 200 µg/mL concentration, methanolic and aq. fruit extracts of Ashwagandha showed H_2O_2 radical scavenging activity of 152.40% and 112.07% respectively. Whereas at 25 µg/mL concentration, methanolic and aq. fruit extract of Ashwagandha showed H_2O_2 radical scavenging activity of 1.95% and 1.35% respectively (Table 2).

Table 2: *In-vitro*antioxidant activity of fruit extracts of Ashwagandha by H_2O_2 radical scavenging activity method

Concentration (µg/mL)	Inhibition (%)		
	Standard Ascorbic Acid	Ashwagandha Fruit Extracts	
		Methanol	Aqueous (aq.)
25	15.95 ± 0.89	1.95 ± 0.67	1.35 ± 0.45

50	22.28 ± 0.78	10.98 ± 0.89	8.87 ± 0.29
100	29.05 ± 0.98	18.45 ± 1.18	14.26 ± 1.54
150	50.38 ± 1.03	34.26 ± 0.91	25.92 ± 2.54
200	118.21 ± 0.89	152.40 ± 2.19	112.07 ± 3.42

Values are expressed as Mean ± S.D; n=3

H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results of our study demonstrated that methanolic fruit extracts of Ashwagandha exhibited superior H_2O_2 scavenging activity as compared to aq. extracts of Ashwagandha.

The antioxidant activity of an antioxidant compound has been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[16] Generally, flavonoids are the important class of antioxidants; hence the medicinal plants containing flavonoids and phenolic compounds are repeatedly screened for antioxidant activity. In addition to flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are reported to possess antioxidant activity.[17]Khan et al., reported the presence of flavonoids, alkaloids and triterpenoids in alcoholic extract of *Withania somnifera*.[18]In concurrence with literature findings, in our study substantial amounts polyphenol content was present in the methanolic and aq. extracts of fruit extracts of Ashwagandha, and the reported H_2O_2 scavenging activity would be attributable to polyphenol contentof Ashwagandha extracts.

Conclusion

The results of present preliminary study clearly demonstrated that methanolic fruit extract*of* Ashwagandhapossess superior antioxidant activity than aqueous fruit extract of Ashwagandha. Hence, it could be suggested that methanolic fruit extract of Ashwagandha could be employed for the management of oxidative stress conditions and could be considered for development of natural antioxidant drugs. However, further studies are recommended to elucidate the exact mechanism of action of particular phytochemical responsible for antioxidant activity of Ashwagandha fruit extracts.

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