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Research Paper

ANTI-OXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF ASHWAGANDHA (*Withania somnifera* L.) LEAVES

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Abstract

Ayurvedic medicines have been continuously using Ashwagandha as one of the active ingredients for centuries due to its pleotropic effects namely anti-inflammatory, immuno-modulatory and antistress properties. The present investigation aims to evaluate the antioxidant and free radical scavenging activity of Ashwagandha leaves. To begin with, the various Ashwagandha leaf extracts were prepared in different solvents of varied polarity starting from non-polar to polar in a sequential manner. The maximum yield of 13.7% was obtained in methanol and the same extracts was found to contained maximum total polyphenolic compounds (TPC, 43.93 µg GAE/mg extract).The methanolic extract of Ashwagandha leaf was found to be appreciably effective in scavenging DPPH radical (EC50=197.50 µg), metal chelation (EC50=76.09 µg), hydroxyl radical (EC50=790.63µg), super oxide radical(EC50=117.70 µg) and inhibition of lipid peroxidation (EC50=536.43 µg). Seven polyphenols *viz.* gallic acid (0.17 µg/g), chlorogenic acid (0.70 µg/g), caffic acid (0.57 µg/g), sinapic acid (1.60 µg/g), rutin hydrate (0.17 µg/g), quercetin-3-rhamnoside (1.61 µg/g) and quercetin (0.27 µg/g) were identified and quantified using Reverse Phase-High Pressure Liquid Chromatography. In conclusion, the study suggests that Ashwagandha leaf extract is of great use for the preparation of antioxidant rich nutraceuticles.

Key words: Ashwagandha, Antioxidant, ROS, Polyphenolics, Oxidative stress.

INTRODUCTION

Oxidative stress plays an important role in the pathogenesis of ageing, inflammation and cancer [1]. Free radicals such as superoxide, hydroxyl radical ($OH^{\cdot}$), nitric oxide, hydrogen peroxide, etc collectively known as reactive oxygen species (ROS) are generated through oxidative stress and have been implicated in etiology of various age related diseases such as atherosclerosis, asthma, stroke, vasospasms, liver damage and Alzheimer's disease etc [2]. Exposure to ionizing radiations also generates ROS, which have been identified as important chemical mediators that regulate signal transduction. These free radicals also affect mitochondrial membrane potential

(MMP) and thus causes apoptosis [3]. The growing number of evidences indicates that these ROS are also responsible for exercise-induced protein oxidation and contribute significantly to muscle and mental fatigue. It has been postulated that treatments that reverse these ROS generated injuries may be acting through mechanisms that scavenge these species [4] .

The Indian medicinal plant *Withania somnifera* (L) Dunal (family- solanaceae), commonly known as Ashwagandha, is widely used in herbal medicine for stress, arthritis, inflammations, conjunctivitis and tuberculosis.[5-9] The active principles of Ashwagandha, consisting of sitoindisides VII-X and withaferin-A have been shown to exhibit significant antistress and antioxidant effect in rat brain frontal cortex and striatum[10]. Keeping in view of its beneficial antioxidative properties and worldwide consumption present study was conducted to evaluate the antioxidant properties of Ashwagandha leaf based on its ability to scavenge various free radicals.

MATERIALS AND METHODS

Preparation of sample extract

Leaves of Ashwagandha were shade dried, powdered and the antioxidant compounds were extracted by adding solvents namely hexane (H), chloroform (C), ethyl acetate (EA), acetone (A), methanol (M) and water (W) in a sequential manner in increasing order of their polarity. After filtering through folded Whatman No. 1 filter paper, the filtrates in different solvents were recovered and this process was repeated thrice with each solvent. Then, the respective solvents from the filtrates were evaporated in a vacuum rotary evaporator to obtain the yield of different extract. For checking the antioxidant activity, each extract/fraction was dried and re-dissolved in dimethylsulfoxide (DMSO).

Determination of total phenolic content (TPC) and total flavonoids (TF)

The TPC of different extracts was determined by the method of Folin-Ciocalteu using gallic acid as the standard [11]. A calibration curve was made for gallic acid and the results were determined from the regression equation of this calibration curve, which was expressed as gallic acid equivalent (GAE) in µg/mg extract. In brief, to 3 ml of appropriately diluted extract was added 0.5 ml of (50%) Folin-Ciocalteu reagent, followed by incubation at room temperature (10 min) and addition of 2 ml of 7% Na_2CO_3 solution. The mixture was boiled for 1 min, cooled and the absorbance was measured at 650 nm.

The determination of TF was carried out according to the method of Delcour and Varebeke [12]. One ml of appropriately diluted extract was mixed with 5 ml of chromogen reagent (0.1% cinnamaldehyde solution prepared in a cooled mixture of 25 ml of concentrated HCl and 75 ml of methanol). After an incubation of 10 min, the absorbance was measured at 640 nm. The TF content was expressed as catechin equivalents (CE) in µg/mg extract.

DPPH radical scavenging assay

All the extracts were evaluated in terms of their hydrogen-donating or radical-scavenging ability using the stable radical, $DPPH^*$, following the method of Blois [13] with slight modifications. Briefly, the reaction mixture contained 3 ml of appropriately diluted extract in methanol and 0.5 ml of 500 µM methanolic solution of $DPPH^*$. The reaction mixture was allowed to stand in dark at room temperature for 45 min and absorbance was recorded at 515 nm against the methanol blank. A control was taken without plant extracts under identical conditions. The percent free radical scavenging capacity (%RSC) of the extracts was calculated from control and IC_{50} from linear regression analysis. BHA was used as a standard antioxidant.

Metal chelation assay

The chelating effect of various extracts on ferrous ions was determined according to the method of Dinis et al.[14] with some modifications. To appropriately diluted extract (2 ml) was mixed 0.05 ml of 2 mM $FeCl_2$ followed by addition of 0.2 ml of 2 mM ferrozine. The mixture was left to react at room temperature for 10 min before determining the absorbance of the mixture at 562 nm. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated from control without sample under similar conditions. The results were expressed as IC_{50} calculated from linear regression analysis. EDTA was used as a standard antioxidant.

Hydroxyl radical scavenging assay

The assay was performed following the method of Halliwell et al.[15] with slight modifications. The reaction mixture contained 0.1 ml of 28 mM 2-deoxyribose, 1 ml solution of various concentrations of extracts, 0.2 ml of mixture solution containing equal amount of 200 µM $FeCl_3$ and 1.04 mM EDTA, 0.1 ml of H_2O_2 (1.0 mM) and 0.1 ml ascorbic acid (1.0 mM). The mixture was then incubated at 37°C for 1 h and boiled for 20 min with 1 ml of 1% TBA in 50 mM NaOH and 1 ml of 5% trichloroacetic acid in a water bath. After cooling, absorbance of the mixture was measured at 532 nm. The % inhibition was calculated from control without sample under similar conditions. IC_{50} was calculated from linear regression analysis. Gallic acid was used as a standard antioxidant.

Superoxide anion radical scavenging assay

The measurement of superoxide anion scavenging activity of different extracts was based on the modified method of Liu et al.[16]. The method is based on the generation of superoxide radicals in PMS–NADH system by oxidation of NADH and subsequent assay of reduced NBT. In this experiment, the superoxide radicals were generated in 3ml of 16 mM Tris–HCl buffer (pH 8.0) containing 1ml of 150 µM NBT solution, 1ml of 468 µM NADH solution and the sample/extract. The reaction was started by adding 1ml of 60 µM PMS solution to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated using control without sample under similar conditions. IC_{50} were calculated using regression analysis. L-ascorbic acid was used as the standard.

Lipid peroxidation inhibition activity

A modified thiobarbituric acid reactive species (TBARS) assay [17] was used to measure the lipid peroxide formed using egg yolk homogenates as lipid-rich media [18]. Briefly, 0.5 ml of 10% v/v egg homogenate and 0.1 ml extract were taken in a test tube and made up to 1 ml with distilled water. Then, 0.05 ml of 0.07 M $FeSO_4$ was added to induce lipid peroxidation and the mixture was incubated for 30 min. It was followed by addition of 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate (SDS). The resulting mixture was vortexed and heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol was added and the content was centrifuged at 3,000 rpm for 10 min. The absorbance of the upper organic layer was measured at 532 nm. Inhibition of lipid peroxidation (%) was calculated using control without sample under similar conditions. IC_{50} was calculated from linear regression analysis. BHA was used as a standard antioxidant.

Characterization of methanol extract

The most potent methanolic extract was examined for the identification and quantification of individual polyphenols. An analytical HPLC system consisting of a JASCO high-performance liquid chromatograph coupled with a UV/VIS multi-wavelength detector was employed for analysis of this extract. The mobile phase consisted of 0.1% formic acid (solvent A) and methanol (solvent B). The programme/gradient used was: 85% A/15% B (0 min), 20% A/80% B (55 min), 85% A/15% B (60 min). The flow rate was 0.8 ml/min and the injection volume was 20 µl. Absorbance was monitored at 270 nm and the identification of each polyphenol was based on retention time [19].

RESULTS AND DISCUSSION

Ashwagandha has been used since centuries in the Ayurvedic system of medicine to enhance longevity and vitality. The plant is reported to contain several alkaloids, withanolides, a few flavonoids and reducing sugars besides rich in iron [20]. The roots, leaves and fruits of this plant have high antioxidant potential. The involvement of a very complex chemistry of oxidation and anti-oxidation processes clearly suggests that a single testing method is inconclusive to gain comprehensive picture of the antioxidant potential of a given herb. In the present work, therefore, we have used a multi-method approach to judge completely the antioxidant potential of various leaf extracts.

Taking zero percent inhibition in the assay mixture without extract, linear regression equations were developed from a plot between the extract concentration and percentage inhibition of free radical formation/prevention. These equations were employed for the calculation of IC_{50} values (concentration of sample required to scavenge 50% free radicals). Lesser the IC_{50} values more is the antioxidant activity of extract.

Extraction efficiency

Starting from non-polar (hexane) to polar (water), six solvents were used sequentially to extract compounds from Ashwagandha leaves and the corresponding yield is presented in Fig. 1. The experiment revealed that maximum migration of molecules took place in methanol and it was the best solvent for the extraction of compounds as it recorded a maximum yield of 13.7%. On the contrary, a minimum yield of 1.05% was recorded in ethyl acetate fraction. Because methanol is a relatively polar organic solvent compared to other extracting solvents like hexane, chloroform, ethyl acetate and acetone, it can be concluded that compounds present in Ashwagandha leaves are most likely polar in nature. Methanol has earlier also been reported as better extractant for the extraction of compounds since methanol is more efficient in cell wall degradation as compared with other solvents [21].

DPPH scavenging activity

The capacity of a test compound to scavenge free radicals can be judged by its ability to bleach DPPH absorption (517 nm). Hence, DPPH-scavenging activities of the extracts were taken as the parameter to check their antioxidant potential. A dose dependent increase in quenching of free radical was observed for all the extracts. Table 1 summarizes the linear regression equations used for calculation of IC_{50} values of different extracts which are shown in Fig. 2. Methanolic fractions (IC_{50} value 197.5 µg/ml) showed the highest free radical scavenging activity followed by water extract (IC_{50} value 231.3 µg/ml). The hexane fraction was found to least potent as indicated by its highest IC_{50} value (713.24 µg/ml).

Metal chelating activity

Iron is essential for life because it is required for oxygen transport, respiration and the activity of many enzymes. However, it is an extremely reactive metal and causes the oxidative damage to lipids, proteins and other cellular components [22]. Therefore, ability of different extracts to chelate/bind metal ion was tested by a method which is based on chelating of Fe^{+2} by the reagent ferrozine, which is a quantitative formation of a complex with Fe^{+2} [14]. Formation of this complex is disturbed by the other chelating agents, which result in the reduction of formation of red-coloured complex. Therefore, measurement of the rate of reduction of the color allows the estimation of chelating activity of the co-existing chelators. In the present study, all the extracts intervened with the formation of red-coloured complex suggesting that it has chelating effects and captures the ferrous ions before ferrozine. The absorbance of Fe^{+2}-ferrozine complex linearly decreased with the increase in concentration of extracts. The regression equations (Table 2) were generated for the calculation of IC_{50} values (Fig. 3). The result shows that the methanolic extract was most potent as evident by lowest IC_{50} value (76.09 µg/ml). It was followed by ethyl acetate fraction (IC_{50} value 98 µg/ml) and acetone fraction (IC_{50} value 116.4 µg/ml). Like DPPH free radical scavenging activity hexane extract also showed least metal chelation activity with IC_{50} value of 600 µg/ml. The data clearly demonstrate that the methanolic extract of leaf possesses effective capacity for iron binding.

Hydroxyl radical scavenging activity

Among the reactive oxygen species, the hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism [23]. This radical is an extremely reactive oxygen species capable of modifying almost every molecule in the living cells. It can cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. Hydroxyl radicals are also capable of quick initiation of lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids [24-25].

However, due to their high reactivity, the radicals have a very short biological half-life thereby demanding its effective scavenger either to be very efficient towards these radicals or to be present at a very high concentration. In the present study, the ability of all extracts to scavenge these radicals was evaluated by the 2-deoxyribose assay. Hydroxyl radicals were generated by

the Fenton reaction which attacks deoxyribose and degrades it into fragments that react with thiobarbituric acid on heating at low pH to form a pink color. All the extracts were found to show a dose dependent response towards chelation of hydroxyl free radical. The linear regression equations were generated which are summarized in Table 3. The best results were exhibited by the methanolic extract with minimum IC_{50} value of 790.7 µg/ml (Fig. 4).

Superoxide radicals scavenging activity

Although by themselves superoxide radicals are less reactive but they can give rise to toxic hydroxyl radicals thereby damaging cellular macromolecules directly or indirectly with severe consequences [26]. The superoxide radicals have been proved to play crucial roles in ischemia-reperfusion injury [27]. These radicals are also involved in many pathological processes. Thus, scavenging of superoxide radicals would be a promising remedy for this disease.

In this experiment, superoxide radicals were generated by a PMS/NADH system and the scavenging activity of different extracts was assessed by measuring the absorbance at 560 nm [28]. As in other assays, the IC_{50} values were calculated using the regression equations listed in Table 4. Fig. 5 summarizes the IC_{50} values of various extracts against superoxide radical anions. The highest scavenging activity was shown by methanolic extract followed by ethyl acetate extract with IC_{50} values of 119.7 and 138.7 µg/ml, respectively. Hexane extract was least potent with highest IC_{50} value of 531.5 µg/ml.

Lipid peroxidation inhibition activity

It has been suggested that antioxidant activity of an extract is its ability to delay the onset of auto-oxidation by scavenging reactive oxygen species, or its ability to act as chain breaking antioxidants by inhibiting the propagation phase of lipid auto-oxidation [29].

The inhibition of lipid peroxidation induced by $FeSO_4$ in egg yolk was assayed by measuring the lipid peroxidation products such as TBARS. All the extracts of Ashwagandha leaves showed inhibition of lipid peroxidation in a dose dependent manner. The regression equations developed for the calculation of IC_{50} values of all extracts is shown in Table 5 and a comparison of IC_{50} values is shown in Fig. 6. Results showed that all the extracts inhibited TBARS formation in a dose dependent manner with maximum activity shown by methanol (536.4 µg/ml) followed by water extract (707.8 µg/ml).

Total phenolic and total flavonoids contents

Plant phenolics are one of the major groups of compounds which act as primary antioxidants or free radical terminators. It is therefore worthwhile to quantify their content in the extracts under study. These compounds have been identified to possess a wide range of chemical and biological activities including radical scavenging activity. Therefore, the total phenolic and flavonoids content were determined in all the extracts of Ashwagandha leaves. It was also aimed to establish relationships of different antioxidant activities with total phenolics and flavonoids.

The TPC values of the different extracts determined using the Folin–Ciocalteu assay, ranged from 7.55 to 43.93 µg GAE/mg of extract (Table 6). This difference was observed due to the varied polarity of the extracting solvent. Among all the fractions, methanol extract was found to have the highest phenolic content (43.93 µg GAE/mg). The lowest amount of phenolics i.e. 7.55 µg GAE/mg was reported in the hexane fraction. The above findings clearly indicate that most polyphenolics evaluated in this study are likely polar compounds.

The flavonoids, another class of secondary plant phenolics with powerful antioxidant properties [30], were estimated in the different extracts under observation. The investigations demonstrated the presence of maximum and minimum flavonoids in ethyl acetate (1.21 µg CE/mg of extract) and hexane (0.18 µg CE/mg of extract) extracts, respectively.

Maximum radical scavenging activity was found in methanolic extract and the same extract also contained maximum phenolic contents. Therefore, a positive correlation between the content of antioxidant compounds and the RSC can be established [31]. The observed data are in coherence with others reports, where it has been shown that high TPC increases the antioxidant activity [32-33] and there is a positive correlation between phenolic content and antioxidant activity [34].The antioxidant activity of phenolic compounds is mainly contributed by their redox potential, which plays an important role in neutralizing free radicals [35].

Characterization of methanol extract

The methanol extract of Ashwagandha leaf was found most potent and an attempt was made to identify the main individual polyphenolic compounds present in it. Quantification was done using RP-HPLC through 'external standard method' *via* calibration with standards. Seven polyphenols namely Gallic acid (0.17 µg/g), Chlorogenic acid (0.70 µg/g), Caffic acid (0.57 µg/g), Sinapic acid (1.60 µg/g), rutin hydrate (0.17 µg/g), Quercetin-3-rhamnoside (1.61 µg/g) and Quercetin (0.27 µg/g) were identified and quantified (Fig. 7 , Table 7).However, few peaks could not be identified due to the lack of standards.

Table 1. Regression equations and correlation coefficients for DPPH scavenging activity of different extracts of Ashwagandha leaves.

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.0687x + 1	0.9884
Chloroform	y = 0.1015x + 0.2333	0.9898
Ethyl acetate	y = 0.1629x + 0.6857	0.9768
Acetone	y = 0.1141x + 3.025	0.9899
Methanol	y = 0.2407x + 2.4611	0.9703
Water	y = 0.2028x + 3.0964	0.9832

Table 2. Regression equations and correlation coefficients for metal chelating activity of different extracts of Ashwagandha leaves.

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.0774x + 3.5622	0.9875
Chloroform	y = 0.2535x - 0.0114	0.9906
Ethyl acetate	y = 0.516x - 0.5917	0.9887
Acetone	y = 0.459x - 3.4417	0.9875
Methanol	y = 0.6837x - 2.0286	0.9881
Water	y = 0.4144x - 3.2117	0.9888

Table 3. Regression equations and correlation coefficients for hydroxyl radical scavenging activity of different extracts of Ashwagandha leaves.

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 7.1971x + 0.581	0.9868
Chloroform	y = 15.737x - 1.325	0.9859
Ethyl acetate	y = 0.0366x - 1.9714	0.9892
Acetone	y = 0.041x + 1.8	0.9849
Methanol	y = 0.0668x - 2.8143	0.9913
Water	y = 24.464x + 1.4321	0.9865

Table 4. Regression equations and correlation coefficients for superoxide radical scavenging activity of different extracts of Ashwagandha leaves.

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.0934x + 0.3619	0.9917
Chloroform	y = 0.2028x + 3.8057	0.9803
Ethyl acetate	y = 0.3307x + 4.1138	0.9846
Acetone	y = 0.2653x - 1.26	0.9906
Methanol	y = 0.3627x + 6.5838	0.9729
Water	y = 0.285x + 3.9446	0.9835

Table 5. Regression equations and correlation coefficients for anti-lipid peroxidation activity of different extracts of Ashwagandha leaves.

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 19.629x + 1.9762	0.9858
Chloroform	y = 0.0406x + 1.6286	0.9884
Ethyl acetate	y = 0.0627x - 0.0771	0.9897
Acetone	y = 0.0508x - 0.1714	0.9802
Methanol	y = 0.0959x - 1.4433	0.9883
Water	y = 0.0677x + 2.0821	0.9877

Table 6: TPC and TF in different extracts of Ashwagandha leaf

Extract	TPC (µg/mg extract)	TF
Hexane	7.55	0.94
Chloroform	16.97	0.98
Ethyl acetate	36.24	1.21
Acetone	20.28	0.33
Methanol	43.93	0.18
Water	26.48	0.13

Table 7: Individual polyphenol of methanolic extract of Ashwagandha leaf

Polyphenols	Amount (µg/g)
Gallic acid	0.17
Chlorogenic acid	0.70
Caffic acid	0.57
Sinapic acid	1.60
Rutin hydrate	0.17
Quercetin-3-rhamnoside	1.61
Quercetin	0.27

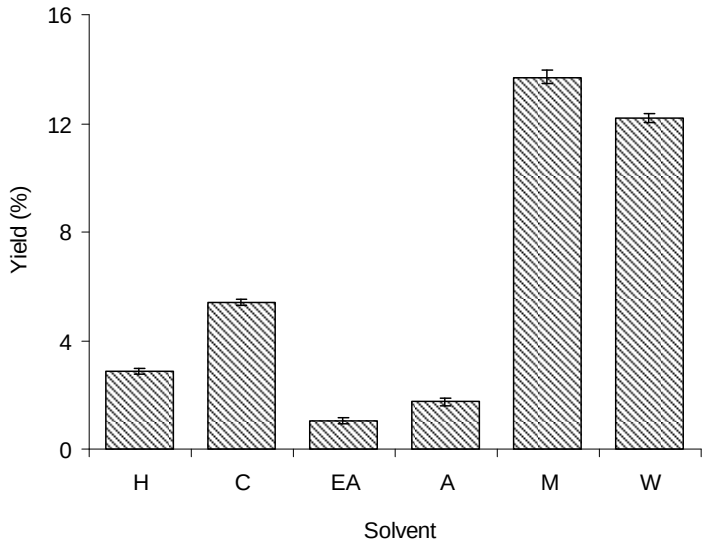

Fig.1. Yields of Ashwagandha leaves compounds in different solvents

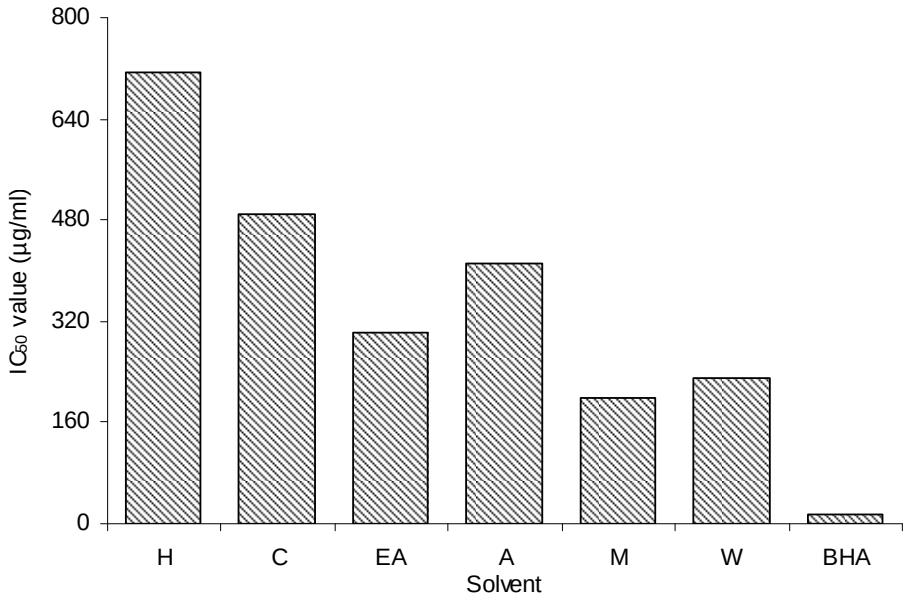

Fig.2. Comparison of DPPH radical scavenging activity of different extracts of Ashwagandha leaves

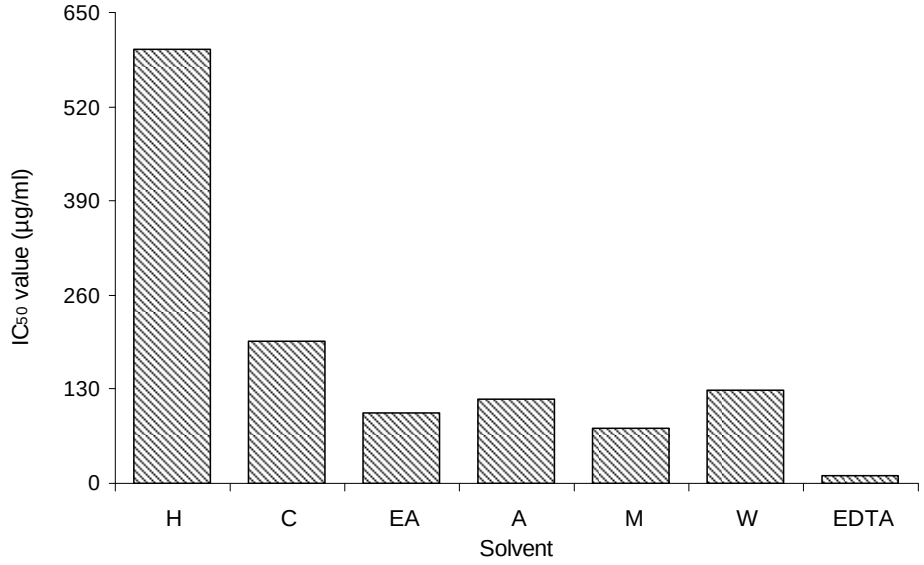

Fig.3. Comparison of metal chelating activity of different extracts of Ashwagandha leaves

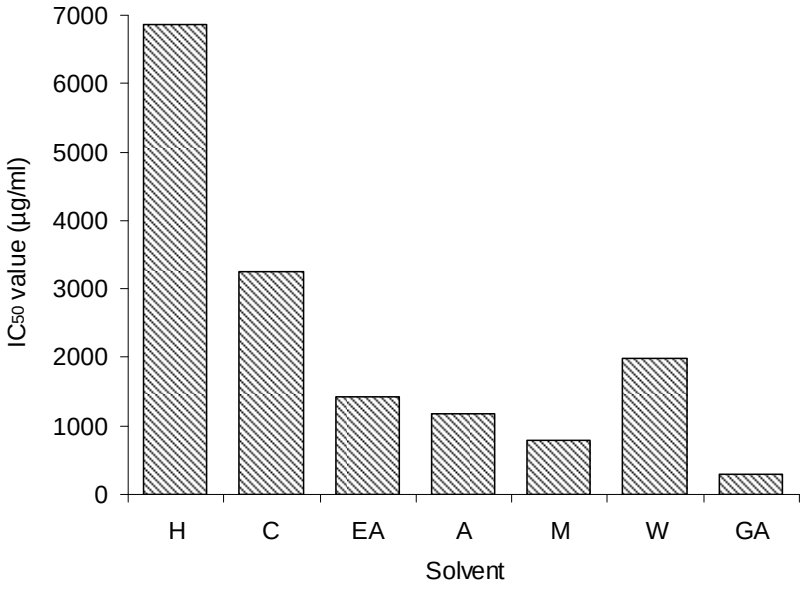

Fig.4. Comparison of hydroxyl radical scavenging activity of different extracts of Ashwagandha leaves

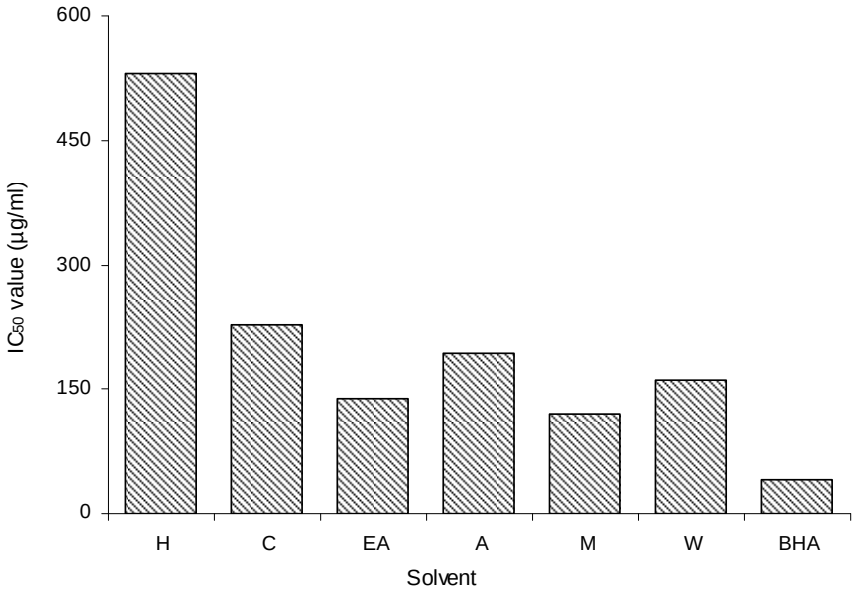

Fig.5. Comparison of superoxide radical scavenging activity of different extracts of Ashwagandha leaves

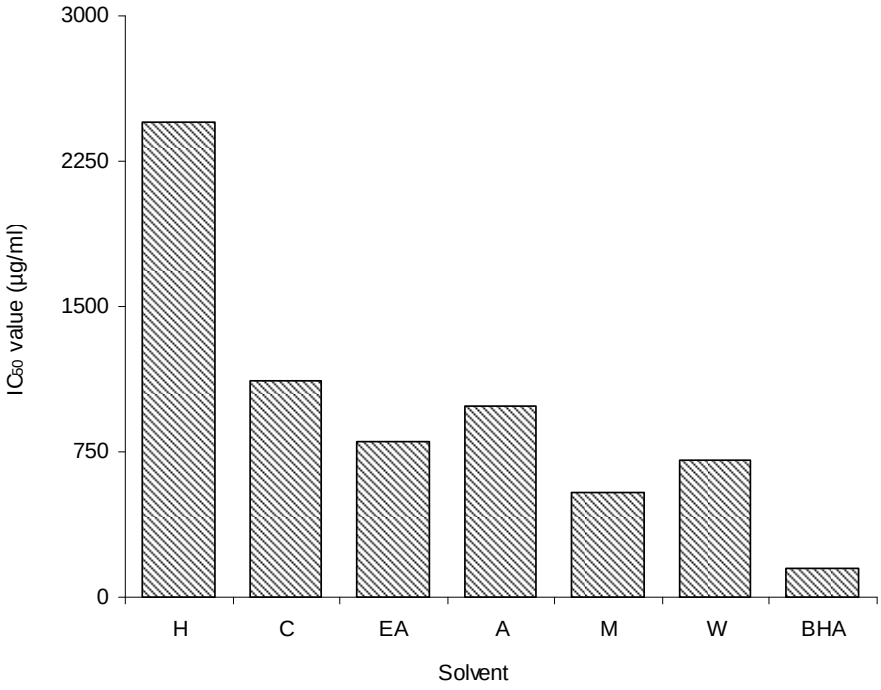

Fig.6. Comparison of anti-lipid peroxidation activity of different extracts of Ashwagandha leaves

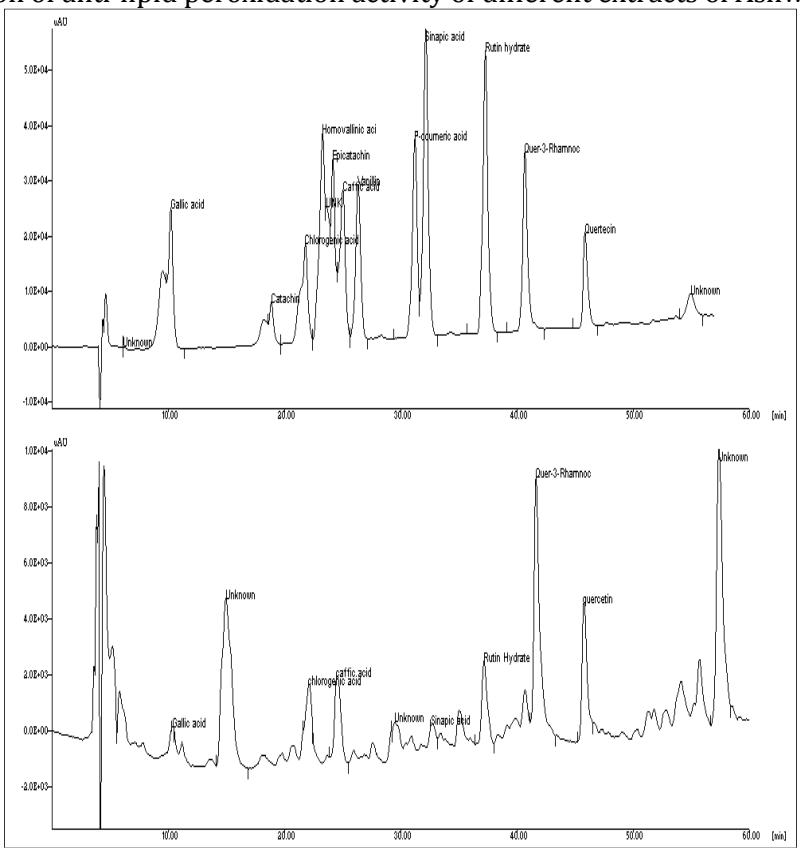

Fig.7. **HPLC chromatograph of Ashwaghand leaf extarct**

CONCLUSION

Solvents of varied polarity starting from non-polar to polar were used sequentially to extract the compounds from Ashwagandha leaves. The methanolic extract was found enriched with total polyphenolic content. Among all the extracts, methanol extract was the most effective in terms of its radical scavenging activities tested using an array of assays. The antioxidant activity

of various extracts can be directly related with the total polyphenolic compounds contain in them. Overall, the present investigation indicates that the methanolic extract is most potent regarding its antioxidant potential.

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