ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research
Available Online at www.jbpr.in
CODEN: - JBPRAU (Source: - American Chemical Society)
Volume 3, Issue 6, 2014, 35-42

RESEARCH ARTICLE

INVITRO ANTIOXIDANT ACTIVITY AND HPTLC ANALYSIS OF *WITHANIA SOMNIFERA*

***Makhija Manju[1], Gupta Maheshkumar K[2]**

[1]*M.Pharm, Rajasthan University of Health Science, Kumbha Marg , Pratapnagar, Jaipur, Rajasthan, India*

[2]*Ph.D, Principal, Kota college of Pharmacy, Kota,Rajasthan,India*

Received 10 October 2014; Accepted 03 November 2014

ABSTRACT

Withania *somnifera* is a well known medicinal plant having wide range of medicinal properties. HAEWS, MEWS and AEWS were comparatively standardized for the content of Withanolide D by HPTLC. All the samples were screened for the possible in-vitro antioxidant activity for 1,1-diphenyl-2-picryl-hydrazil (DPPH˙) free radical scavenging, hydrogen peroxide scavenging, flavonoid content, polyphenolic contents, and lipid peroxidation effects. The Withanolide D content of HAEWS, MEWS and AEWS was found to be 0.103, 0.067, and 0.036% (w/w) respectively. The Polyphenol and flavonoid content of HAEWS was found to be 35.51, 17.68 (w/w) respectively. All the tested samples showed marked antioxidant activity in which HAEWS showed prominent activity. HAEWS showed significant ($p<0.01$) (DPPH) free radical scavenging (67.38%), hydrogen peroxide scavenging (61.05%) and lipid peroxidation (63.86%) , reducing power (0.93%) at the concentration of 1000µg/ml. These various antioxidant activities were compared to standard antioxidant such as ascorbic acid.

Key words: Withania *somnifera*, HPTLC, antioxidant, radical scavenging.

Abbreviations:
HAEWS: Hydroalcholic extract of Withania somnifera
MEWS: Methanolic extract of Withania somnifera
AEWS: Aqueous extract of Withania somnifera
DPPH: (1,1-Diphenly-2-picryl-hydrazil)

INTRODUCTION:

Withania somnifera (Dunal), also known as Ashwagandha, is an important member of family Solanaceae. In Ayurveda, ashwagandha is classified as a rasayana (rejuvenation) and acknowledged to increase longevity and vitality [1].

Ashwagandha is one of the major ingredients in a variety of Siddha, Ayurveda and Unani formulations prescribed for possessing antiinflammatory, antistress, antioxidant, immunomodulatory, antiparkinsonism, antibacterial, rejuvenating and antitumor properties[2].

Withaferin A and withanolide D are the two main withanolides contribute to the most of the biological actions of withania [3].

The antioxidative properties of some vegetables and fruits are partly due to the low molecular weight phenolic compounds, which are known to be potent as antioxidants [4]. To evaluate antioxidant activities of compounds and of complex mixtures such as plant extracts various methods have been developed [5]. Just one procedure cannot identify all possible mechanisms characterizing an antioxidant activity [6]. Therefore, the aim of this study is to evaluate the anti-oxidative activity of *Withania somnifera* root using several different methods, and to evaluate the relationship between the anti-oxidative activity and content of the plants.

HAEWS was standardized using high performance thin layer chromatography for the content of withanolide D. Along with this methanolic extract and aqueous extract were also standardized. An attempt was made to compare the in-vitro antioxidant potential of the prepared HAEWS, MEWS and AEWS.

Materials and Methods

Procurement of *Withania somnifera* roots

Authenticated Withania somnifera roots were purchased from NRIBAS, Pune, India.

Preparation of Hydroalcholic extract of Withania somnifera (HAEWS)

**Corresponding author: Makhija Manju | E-mail:* manjumakhija12@gmail.com

The dried roots were coarsely powdered, weighed and filled in Soxhlet apparatus for extraction. The solvent used was hydroalcholic i.e. 50% ethanol and 50% water. Percent yield was calculated for each extract after drying

Methanolic extract of Withania somnifera (MEWS)

50 gm of root powder was extracted with 250 ml of methanol in a soxhlet apparatus for 18 hrs.

Aqueous extract of Withania somnifera (AEWS)

WS root powder (50) was dispersed in 250 ml water. The mixture was kept in incubator shaker at 150 rpm for 24 hrs at 60°C. The extract so prepared was in the form of free flowing powder. But it was found to be highly hygroscopic in nature. Hence it was stored in airtight containers in the refrigerator.

Chromatographic conditions

Samples were spotted as bands (width, 6 mm) with a CAMAG microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm with 250 µm thickness, E. Merck, Germany) using a CAMAG Linomat IV spotter (Switzerland). A constant application rate (0.1 µl/s) was employed and space between two bands was 6 mm. Mobile phase consisted of Toluene, ethyle acetate, and formic acid (4:6:0.1 v/v/v). Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. Optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 230 nm. Slit dimension was kept at 6 mm x 0.45 mm, and 10mm/sec scanning speed was employed. A model III TLC scanner with CATS IV integration software was used. The source of radiation utilized was deuterium lamp.

Standard solution and calibration curve

Stock solution of Withanolide D containing 1mg/ml was prepared in methanol and aliquot of 1ml of the above stock solution was transferred to a 10 ml volumetric flask and diluted to volume with methanol to obtain a working stock solution of 100 µg/ml. Calibration curve was obtained by applying different aliquots (2-20 µl) of working stock solution (100 µg/ml) in the range of 200-2000 ng/spot in triplicate for Withanolide D. Calibration curve was constructed by plotting the peak area versus analyte standard solutions at various concentrations. The plate was developed with a solvent system consisting of toluene: ethyl acetate: formic acid (4:6:0.1 v/v) and densitometric scanning was performed in the absorbance mode at 230nm.

Chemicals and Reagents

α-tocopherol, nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxy toluene (BTA), l- ascorbic acid, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), polyoxyethylenesorbitan monolaurate (Tween – 20) and trichloroacetic acid (TCA) were obtained from Sigma Aldrich, US. All other chemicals used were analytical grade and obtained from Merck, US.

Antioxidant assays

Different biochemical assays were undertaken to assess the antioxidant activity of *WS* roots extract

Free radical scavenging activity by DPPH method

The free radical scavenging activity was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH$^{\cdot}$) using the method described by Shimada et al. [7]. DPPH (1, 1- diphenyl- 2-picryl- hydrazyl) is stable free radical and methanolic solution of it is used to evaluate the antioxidant activity of several natural compounds. Antioxidants on interaction with DPPH, either transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character, and convert it to 1,1- diphenyl- 2-picryl- hydrazine and the degree of discoloration indicates the scavenging activity of the drug. The change in the absorbance produced at 517 nm, has been used as a measure of antioxidant activity. 1ml Different concentration of extract solution and standard were taken in different vials. To this 5 ml of methanolic solution of DPPH was added, shaken well and mixture was incubated at 37 ^{0} C for 20 min. Measure the absorbance against methanol as blank at 517nm. Take absorbance of DPPH as control.

Percent antiradical activity can be calculated by using following formula.

% Anti-radical activity = (Control Abs - Sample Abs x 100 / Control Abs)

Reducing power assay

Total reduction capability was estimated by using the method of Oyaizu [8].The reducing capability was measured by the transformation of Fe3+- Fe2+ in the presence of different extracts at 700nm as per the reported method**.** Increased absorbance of the reaction mixture indicates increased reducing power.

Procedure

1ml of different concentrations of extracts (250-2500 mg/ml) was mixed with 2.5 ml of phosphate buffer and 2.5 ml of potassium ferrocyanide. The mixture was incubated at 500C for 20 min. 2.5 ml of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. 2.5 ml of upper layer solution was taken and mixed with 2.5 ml distilled water

and 0.5 ml of $Fecl_3$ solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Hydrogen peroxide scavenging assay

The ability of extract to scavenge H_2O_2 was determined according to the method of Ruch *et al*. **[9]** A solution of H_2O_2 was prepared in PBS (pH 7.4). H_2O_2 concentration was determined spectrophotometrically, by measuring absorption with extinction coefficient for H_2O_2 of 81m-1cm-1 Hydrogen peroxide (H_2O_2) is generated in vivo, under physiological conditions by peroxisomes, by several oxidative enzymes including glucose oxidase and d-amino acid oxidase, and by dismutation of superoxide radical, catalysed by superoxide dismutase. One of the most common methods for assessing the scavenging capacity against this molecule is based on the intrinsic absorption of H_2O_2 in the UV region. As the H_2O_2 concentration is decreased by scavenger compounds, the absorbance value at 230 nm is also decreased. Nevertheless, it is quite usual that samples also absorb at this wavelength, requiring the performance of a "blank" measurement. The antioxidants can inhibit the reaction by (a) reacting directly with H_2O_2, (b) reacting with intermediates formed from enzyme.

Extracts (100-500 mg/ml) in distilled water were added to H_2O_2 solution (0.6 ml, 10 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2. The percentage of H_2O_2 scavenging of both the extract and standard compound were calculated as follows:

H_2O_2 Scavenged (%) = (A cont. - A test x 100 / A cont.)

Where, A cont. = Absorbance of control reaction

A test = Absorbance of test reaction

Anti-lipid peroxidation effect

Lipid peroxidation in rat liver and brain homogenate was evaluated by the TBA method [10].Decomposition of lipid membrane in the body leads to the formation of Malondialdehyde (MDA) along with other aldehydes and enals as the end product.

These react with thiobarbituric acid to form colored complexes. Hence these are called as the Thiobarbituric Acid Reactive Substances (TBARS). The complex of TBA-MDA is selectively detected at 532 nm using UV spectrophotometer.

The mixtures containing 0.5 ml of homogenate, 1 ml of 0.15 M Kcl, and 0.5 ml of different concentrations of drug extract were prepared. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride. The reaction mixtures were incubated for 30 min at 37°c. After incubation, the reaction was stopped by adding 2 ml of ice-cold 0.25 N HCl containing 15% trichloroacetic acid (TCA) & 0.38% thiobarbituric acid (TBA), and 0.2 ml of 0.05% butylated hydroxy toluene (BHT). These reaction mixtures were heated for 60 min at 80°C, cooled and centrifuged at 5000 g (≈ 6900 rpm) for 15 min. The absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except liver homogenate & drug. Identical experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug) lipid Peroxidation level in the tissue. The percentage of anti-lipid Peroxidation effect (% ALP) was calculated by the following formula:

% ALP = ($FeCl_3$O.D.–Sample O. D.) ×100 / $FeCl_3$ O.D. – Normal O.D

Total Phenolic content

The total phenolic content in the samples was measured according to the method described by Harish and shivanandappa [11]. One ml of the extract was added to 10 ml of distilled water and 2 ml of Folin-phenol reagent. The mixture was then allowed to stand for 5 min and 2 ml of sodium carbonate was added to the mixture. The absorbance was measured at 765 nm in a spectrophotometer. Phenolic content was estimated using gallic acid as the standard and expressed as µg of gallic acid equivalent per g of extract.

Total Flavonoid content determination

The total flavonoid content was determined using the method of Meda et al [12]. In brief, 5mL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the equal volume of the extract solution. After 10 minutes absorption was measured spectrophotometrically at 415 nm against a blank sample consisting of a 5 mL extract solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with quercetin (100-1000 µg/L) as the standard. The result is expressed as µg of quercetin equivalents/g of extract.

Statistical analysis

All the data are expressed as mean ± standard error of the mean (SEM). IC_{50} values were calculated using linear regression analysis from the regression equation obtained using standard calibration curve. One way ANOVA followed by Dunnett's multiple comparison tests was performed. $p<0.05$ was considered to be significant.

Results

Standardization

Quantitative estimation of withanolide D content in different WS extracts

Quantitative estimation of withanolide D content in different WS extracts (Hydroalcoholic extract, aqueous and methanolic extract) was carried out and the results are summarized in table 1. The representative densitograms for different extracts are shown in fig 1, 2 &

3. The study shows that withanolide D content is maximum with HAEWS than with other extracts.

Aqueous extracts contain only hydrophilic constituents and not the lipophilic constituents; thus there occurs loss of many constituents from the drug and extractability becomes poor. These extracts even on drying produce sticky mass, which becomes a major hurdle in further processes of developing of extract in to formulations. The aqueous extracts also suffer from problem of microbial growth and the presence of moisture in the dried extract may result in degradation of active constituents.

Table 1: % withanolide D in all the extracts

Sr no	Extract	% withanolide D
1	AEWS	0.036±0.01
2	MEWS	0.067±0.015
3	HAEWS	0.103±0.01

Data given is mean + SD (n=3)

DPPH Scavenging Assay

A significant ($p<0.01$) decrease in the concentration of DPPH radical was exhibited by all the extract and standards. The scavenging effect of the samples was concentration dependent. The scavenging effect of the samples on the DPPH radical decreased in the order of Ascorbic acid >HAEA>MEA>AEA and were concentration dependent. The IC_{50} value of the HAEWS, MEWS, and AEWS were 0.55, 0.64, and 0.73 respectively. The free radical scavenging activity was increased with increase in concentration.

The free radical scavenging activity was increased with increase in concentration.

Reducing Power assay

All the tested concentrations of the samples showed higher reduction capability. HAEA showed higher reduction capability. The reducing power of HAEWS, MEWS and AEWS increased with increasing concentration. The results were shown in table 4. The reducing power exhibited by the samples were statistically significant ($p<0.01$) and followed the order HAEA >MEA>AEA respectively.

Hydrogen peroxide scavenging assay Ascorbic acid, used as a standard, at 10 µg/ml concentration showed 97.28% inhibition, Hydrogen peroxide scavenging activity of the extract was concentration-dependent, about 72.40% H_2O_2 scavenging activity at 1000 µg/ml concentration was observed indicating that HAEA is good scavenger of peroxide radicals.

Anti-Lipid Peroxidation Assay

Inhibitory effects of ascorbic acid and all other extracts in the concentration range 100 to 1000 µg/ml. It is evident that the HAEWS exhibited prominent anti lipid peroxidation (63.86%) than MEWS (48.5%) and AEWS (32.69%) with the IC_{50} values of 0.71, 1.03 and 1.54 respectively.

Polyphenolic and flavonoid content

The presence of phenolic and flavonoids always correlated with the antioxidant activity. Phenolic compounds and flavonoids are considered to play a major role in the antioxidant activity of the plants. So the extraction of these components is a valuable task in determining the antioxidant property. The total phenolic content and flavonoid content of the samples were shown in table 6 &7 respectively**.** Our findings suggest that HAEWS is rich in polyphenol and flavonoid composition. This in turn suggests the HAEWS is effective in extracting both hydrophilic as well as lipophilic components from WS.

Conclusion

The HAEWS was proved to possess high antioxidant potential than the other extracts. The study shows that withanolide D content is maximum with HAEWS than with other extracts. The polyphenol and flavonoid content is maximum with HAEWS than with other extracts. It shows that though polyphennnol and flavonoid are responsible for the antioxidant property of WS , the high antioxidant activity may be due to synergistic effect by withanolides which were better extracted with HAEWS. The antioxidant property of WS is a part of mechanism for the medicinal effects of WS.

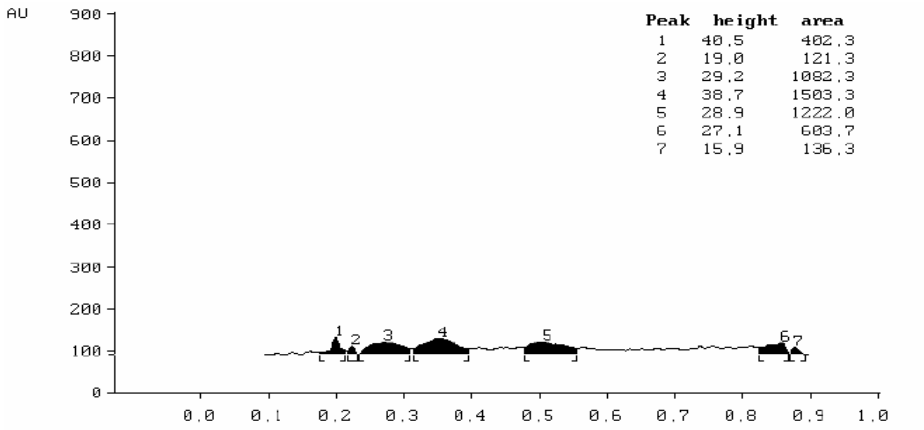

Figure 1: Densitogram of aqueous extract of Withania somnifera root(AEWS).

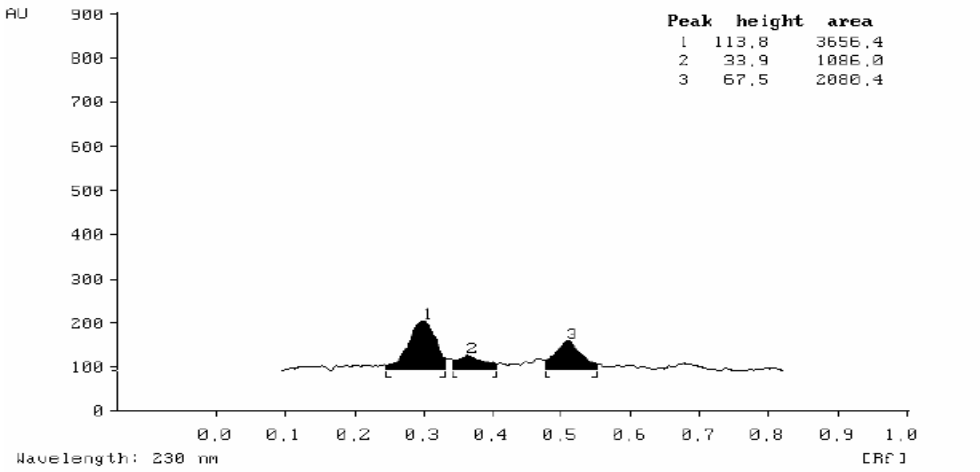

Figure 2 : Densitogram of methanolic extract of Withania somnifera root (MEWS).

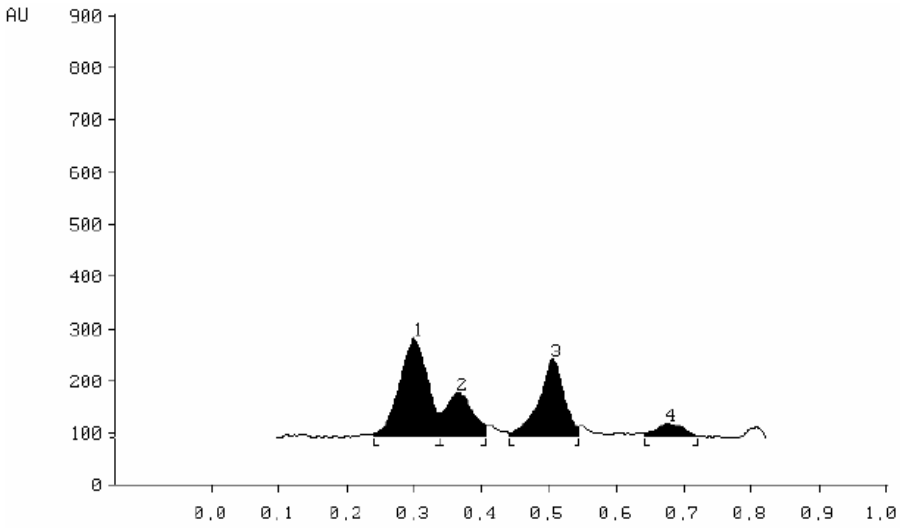

Figure 3 : Densitogram of Hydroalcoholic extract of Withania somnifera (HAEWS).

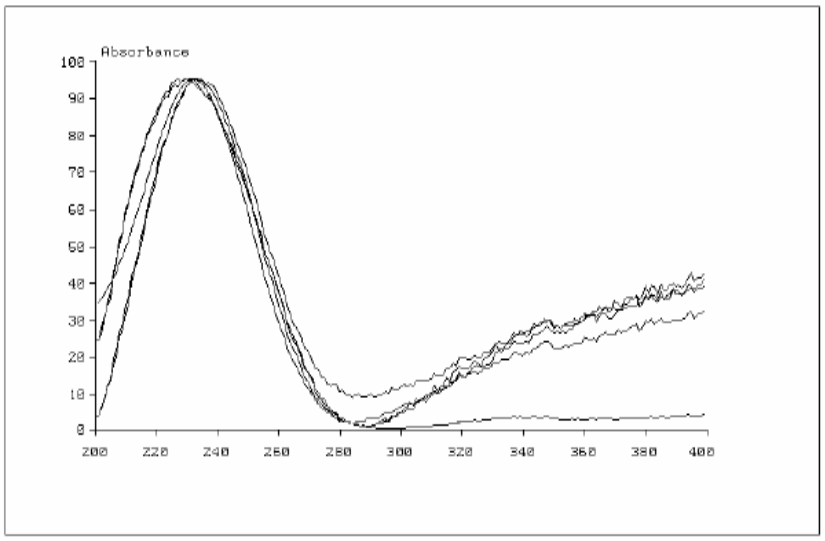

Figure 4: Overlapping spectra of std (withanolide D) and all other extracts of WS.

Table 1: DPPH scavenging activity of various extracts of WS

Concentration (µg/ml)	% scavenging activity			
	AEWS	HAEWS	MEWS	Ascorbic acid
100	13.8±6.00	26.58±7.31	22.08±5.01	74.12±0.72
200	19.06±5.71	31.42±8.53	27.06±6.13	75.58±0.13
300	27.46±0.64	38.05±6.78	33.35±5.48	78.20±1.07
400	34.70±0.84	43.60±3.76	39.20±4.41	78.94±0.67
500	44.56±1.21	54.04±5.77	49.48±5.09	89.18±1.25
1000	61.44±2.6	67.38±6.04	64.44±5.37	94.95±1.31

Data given is mean + SD (n=3)

Table 2: Reducing power of various extracts of WS.

Concentration (µg/ml)	Mean absorbance ± S.D.			
	AEA	HAEA	MEA	Ascorbic acid
100	0.23 ±0.05	0.24 ±0.02	0.24 ±0.04	0.97 ±0.04
200	0.25 ±0.05	0.32 ±0.06	0.28 ±0.03	1.30 ±0.008
300	0.31 ±0.05	0.40±0.05	0.36 ±0.10	1.62±0.02
400	0.38 ±0.05	0.52 ±0.07	0.60 ±0.07	1.96±0.05
500	0.54 ±0.04	0.64 ±0.05	0.78 ±0.04	2.25 ±0.23
1000	0.69 ±0.02	0.93 ±0.05	0.90 ±0.09	-

Table 3: H2O2 scavenging activity of various extracts of WS.

Concentration (µg/ml)	% scavenging activity			
	AEA	HAEA	MEA	Ascorbic acid
100S	6.39±7.6	16.83±7.2	22.93±0.1	79.58±4.1
200	16.25±4.5	18.52±10	26.02±3.6	86.68±1.8
300	22.71±6.6	27.52±7.1	31.26±2.8	90.08±2.3
400	29.29±6.4	32.36±4.7	37.27±3.5	94.51±1.6
500	34.02±4.3	50.15±4.6	43.68±6.6	96.10±1.6
1000	40.43±7.1	61.05±3.9	54.30±202	97.28±0.6

Data given is mean + SD (n=3)

Table 4: Anti-lipid peroxidation in liver homogenate.

Concentration (µg/ml)	% scavenging activity			
	AEA	HAEA	MEA	Ascorbic acid
100	8.05±3.38	11.17±6.2	18.37±0.6	66.31±2.1
200	12.43±3.68	13.97±7.9	24.17±0.8	70.57±0.3
300	16.88±6.3	25.58±6.9	28.25±0.2	78.23±3.1
400	20.96±3.4	34.24±0.4	28.14±8.5	80.40±1.9
500	26.58±5.8	43.37±0.03	35.65±4.5	83.60±0.3
1000	32.69±8	63.86±0.06	48.50±2.9	86.14±1

Data given is mean + SD (n=3)

Table 5: Total flavonoid and polyphenol content

Samples	Polyphenolic Content (mg/g), mean ± SE	Flavonoid content (mg/mg), mean ± SE
AEWS	-	-
MEWS	31.88	15.72
HAEWS	35.51	17.68

REFERENCES:

1. S. Mathura, P. Kaura, M. Sharmaa, A. Katyala, B. Singhb, M. Tiwaria, R Chandraa, The treatment of skin carcinoma, induced by UV B radiation,using 1-oxo-5b, 6b-epoxy-witha-2-enolide, isolated from the roots of Withania somnifera, in a rat model Phytomedicine 11, 452-460.
2. Bhatnagar. M, Jain C.P, Sisodia S.S; 2005. Anti ulcer activity of Withania somnifera in stress plus pyloric ligation induced Gastric ulcer in Rats., Journal of cell and tissue research. 5 (1), 287-292.
3. Sumantran.V.N, Harsulkar.A., 2007 Chondroprotective potential of root extracts of *Withania somnifera* in osteoarthritis; J. Biosci. 32; 299–307.
4. H Wang, M.G. Nair, G.M. Strasburg, A.M. Booren, J.I. Gray, 1999, Antioxidant polyphenols from tart cherries (Prunus cerasus), Journal of Agricultural and Food Chemistry, 47(3), 840-844.
5. M Antolovich, P.D. Prenzler, E. Patsalides, S. McDonald, and K. Robards, 2002, Methods for testing antioxidant activity, The Analyst, 127(1), 183-198.
6. EN Frankel, and A.S. Meyer, 2000, The problems of using one-dimensional methods to evaluate

multifunctional food and biological antioxidants, Journal of the Science of Food and Agriculture, 80(13), 1925-1941.

7. Shimada, K., Fujikawa, K., Yahara, K., Nakamura, T. 1992. Antioxidative properties of xanthone on the auto oxidation of soybean in cylcodextrin emulsion. J. Agric. Food Chem. 40, 945 – 948.

8. Oyaizu M. (1986) Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutr* **44:** 307 – 15.

9. Ruch RJ, Cheng SJ, Klaunig JE. 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 10(6), 1003 – 1008.

10. Ohkawa H, Ohishi N, Yagi K, 1979. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal Biochem,* 95, 351–358

11. Harish, R and shivanandappa, T.,2006. Antioxidant activity and hepatoprotective potential of Phyllanthus niruri. Food Chemistry 95, 180-185.

12. Meda, A., Lamien, C.E., Romito, M., Millogo, J., Nacoulma, O., G. 2005. Determination of the total phenolic, flavonoid and proline contents in burkina fasan honey, as well as their radical scavenging activity. *Food Chemistry,* 91, 571–577.

