Asian J. Exp. Sci., Vol. 22, No. 3, 2008; 213-220

Antioxidant and Free Radical Scavenging Properties of Developed Mono- and Polyherbal Formulations

P.K. Jain and R.K. Agrawal*
Pharmaceutical Chemistry Research Laboratory,
Department of Pharmaceutical Sciences,
Dr. Hari Singh Gour University, Sagar (M.P.); India

Abstract : The antioxidant properties of developed mono and polyherbal tablet formulations *viz.* Withatab, Asparatab, Centab and Ascenwit that probably involve free radical mechanisms, were evaluated by the methods, namely the DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay, Super oxide scavenging assay, Nitric oxide radical scavenging assay and ABTS (2, 2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) radical scavenging assay. The standardized methanol extract from *Withania somnifera*, *Asparagus racemosus* and *Centella asiatica* was used for formulation of tablets. DPPH and Superoxide radical scavenging activity of the formulations was in order of Ascenwit > Centab > Asparatab> Withatab. The Nitric oxide radical activity was found in order of Ascenwit > Withatab > Centab > Asparatab. The Ascenwit was most active in the ABTS assay with an IC_{50} value of 99.56 µg/mL followed by Centab and Withatab with IC_{50} value of 188.39 µg/mL and 293.48 µg/mL. The minimum ABTS radical scavenging activity showed by Asparatab with IC_{50} value of 391.61 µg/mL. The present studies suggest that all the developed formulations have moderate to potent antioxidant activity. In comparison to individual formulations, Ascenwit was superior antioxidant in all the models tested.

Key words : Antioxidant activity, Ascenwit, ABTS radical, DPPH radical.

Introduction

The traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. Experiment evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases (Richards and Sharma, 1991; Niwa, 1991). As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Halliwell, 1994; Poulson and Loft, 1998). Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant

* **Corresponding author :** R.K. Agrawal, Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar (M.P.); India; E-mail : *dragrawal2001@yahoo.co.in*

activity (Zheng and Wang, 2001; Cai *et al.,* 2003). The intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing (Mclarty, 1997; Yang *et al.,* 2001; Sun *et al.,* 2002).

Herbal drugs are rapidly becoming popular in recent years as an alternative therapy. Numerous polyherbal formulations, which are combinations of different herbal extracts/fractions, are used for the treatment of liver diseases. Antioxidants that can protect liver from oxidative damages are included in Polyherbal formulations. For developing a satisfactory antioxidant herbal formulation, there is a need to evaluate the formulation for desired properties such as antioxidant activity. The desired activities of the polyherbal formulations containing different plants/extracts have to be tested again in the formulation form (Chandrasekar *et al.,* 2006).

Condensing the importance of this area, the mono and polyherbal tablet formulations [i.e. Withatab (tablet prepared form standardized *Withania somnifera* extract), Asparatab (tablet prepared form standardized *Asparagus racemosus* extract), Centab (tablet prepared form standardized *Centella asiatica* extract)] and combination formulations [Ascenwit (equi-proportion of standardized extracts of *Withania somnifera, Asparagus racemosus* and *Centella asiatica*)] were developed and tested for antioxidant activity. *In vitro* antioxidant activity was done by DPPH free radical scavenging activity assay (Vani *et al.,* 1997). Super oxide scavenging activity assay (Yen and Chen, 1995), Nitric oxide radical scavenging activity assay (Shreejayan and Rao, 1997) and ABTS radical scavenging assay (Auddy *et al.,* 2003).

Material and Methods

The root of the *Withania somnifera* and *Asparagus racemosus* were picked up in the month of February-March from the University Campus of Dr. H.S. Gour Vishwavidyalaya, Sagar (M.P.) and the leaves of the *Centella asiatica* were purchased from the Local Market of Sagar (M.P.), India. The identity of collected / procured drug was confirmed at Botany Department at Dr. H.S. Gour Vishwavidyalaya, Sagar (M.P.).

Methanolic Extract

For the preparation of methanolic extract, 500 gm of coarse powder of each drug was extracted in Soxhlet apparatus with two liters of methanol for Twenty four hours and filtered to yield the extract. The extract was then concentrated and finally dried to a constant weight.

Formulation of Mono and Polyherbal Tablets

Blend

The dry granulation technique was selected because it is always better for small-scale preparations. The standardized extracts and other ingredients for each formula were weighed, mixed and passed through Sieve no. 80 separately. All the materials were mixed together except Talc and Magnesium stearate was milled in a pestle and mortar and passed again through Sieve no. 80. The materials were mixed with the binder solution, which was added little by little. After mixing, the blend was passed through Sieve no. 80 to obtain the granules and they were dried at 35°C in vacuum dryer. After drying, the granules were again passed through Sieve no. 18 to remove bigger granules and stored in desiccators.

Punching

Tablets were punched using hand rotating single punch machine. The punching machine was cleaned properly. The granules were mixed with Talc and Magnesium stearate. The punching machine was adjusted for the required weight (per tablet) and hardness using a small quantity of the blend. After attaining the required tablet parameter, the whole blend was punched into tablets. The weight variation and hardness of punched tablets were monitored frequently.

Antioxidant Activity of Developed Mono and Polyherbal Formulations

Ten tablets of individual and combination formulations were taken and powdered separately in pestle and mortar. The individual powder was dissolved in small quantity of methanol in 100 ml volumetric flask and made up the volume. The sample was filtered through one micron filter paper and the filtrate was subjected for following antioxidant assays viz. DPPH free radical scavenging, super oxide radical scavenging, Nitric oxide radical scavenging, ABTS radical scavenging assay.

DPPH radical scavenging assay

DPPH [1,1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple color, the intensity of which is measured at 510 nm spectrophotometrically. Antioxidants reduces DPPH to 1,1-diphenyl-2-picryl hydrazine, a colorless compound.

Reagents / chemicals used

DPPH (2,2-diphenyl-1-picryl hydrazyl) [RM 2798, Himedia, India], Methanol (HPLC grade) [43602, Qualigens], Positive control (Gallic acid) [G7384, Sigma, USA].

Procedure

Various concentrations (200 µl) of test solution and 50 µl of DPPH (0.659 mM) solution are incubated at 25°C for 20 min. Following which the absorbance is read at 510 nm. A control reaction was carried out without the test sample. Linear graph of concentration vs percentage inhibition was prepared and IC_{50} values were calculated. The % inhibition was calculated according to the following equation

$$\% \text{ Inhibition} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract.

Superoxide radical scavenging activity (PMS-NADH System)

Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitroblue tetrazolium which yields a chromogenic product, which is measured at 560 nm.

Reagents/chemicals used

Reduced nicotinamide adenine dinucleotide Sodium salt (NADH) [RM 393Himedia, India], Phenazine methosulphate (PMS) [5165, Loba Chemie, India], Nitroblue tetrazolium (NBT) [94060, S.d. fine Chemicals, India], Positive control: Gallic acid G7384, [Sigma, USA].

Procedure

Test solution (0.1 mL) in 0.1M phosphate buffer pH 7.4, 62.5 µl of 468 µM NADH solution, 62.5 µl of 150 µM NBT solution and 62.5 µl of 60 µM PMS solution were added to a microwell plate and incubated at room temperature for 5 min. The absorbance was read at 560 nm. Linear graph of concentration vs percentage inhibition was prepared and IC_{50} values were calculated.

Nitric oxide radical scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions, which can be measured at 546 nm spectrophtometrically in the presence of Griess reagent.

Reagents/chemicals used

Sodium nitroprusside [40190, S.d.fine Chemicals, India], Sulphanilamide [3164, NR Chem., India], Orthophosphoric acid [39416, S.d.fine Chemicals, India], N-(1-naphthyl) ethylenediamine [N5889, Sigma, USA], Positive control: Curcuminoids (33533, Synthite, Kochin, India).

Procedure

Test solution of various concentrations (50 µl) and 50 µl of 10 mM sodium nitroprusside are illuminated (using fluorescence light/18W CDL 6500K) at room temperature (25-30°C) for 15 min. Following

incubation, 125 µl of Griess reagent was added and incubated for 10 min at room temperature. The color developed was measured at 546 nm. Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} values were calculated.

ABTS radical scavenging assay

ABTS (2, 2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) 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 which can be followed spectrophotometrically. The relatively stable ABTS radical has a green color and is quantified spectrophotometrically at 734nm.

Reagents/chemicals used

Ammonium persulphate (APS)[Rankem, India], ABTS (2,2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) [Sigma, USA], Positive control: Gallic acid (3,4,5-Trihydroxy benzoic acid)[Sigma, USA]

Procedure

ABTS radical cations were produced by reacting ABTS and APS and incubating the mixture at room temperature in dark for 16 hours. Add 20 ìl of various concentrations of 10 mM PBS pH 7.4 test solutions and 230 ìl of ABTS radical solution (0.238 mM). The absorbance is measured immediately at 734 nm. A control reaction was carried out without the test sample. Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} values were calculated.

Results

In our study mono and polyherbal tablet formulations were prepared using the methanolic extracts of *Withania somnifera*, *Asparagus racemosus* and *Centella asiatica*. Single tablet containing 100 mg of the each extracts with the excipients made upto total of 300 mg was developed they were named as Withatab (Withania extract) (Table 1-3), Asparatab (Asparagus extract) and Centab (Centella extract). Similarly a polyherbal formulation comprising of each extract (100 mg each) with excipients was developed to a total weight of 500 mg named as Ascenwit (Table 4)

All the four formulations were evaluated for antioxidants activity by using the DPPH, superoxide, nitric oxide and ABTS models. Results showed that in comparison to individual formulations, Ascenwit was superior antioxidant in all the models tested.

In DPPH, model Ascenwit exhibited IC_{50} value of 51.96 µg/ml, Withatab showed IC_{50} value of 380.95 µg/ml, Asparatab showed IC_{50} value of 267.47 µg/ml, Centab showed IC_{50} value of 226.72 µg/ml (Table 5). In superoxide model Ascenwit exhibited IC_{50} value of 49.89 µg/ml, Withatab showed IC_{50} value of 299.58 µg/ml), Asparatab showed IC_{50} value of 276.09

Table 1 : Formulation of Withatab

S. No.	Ingredient	Weight of Tablet (300 mg)	Weight for 400 Tablets (gm)
1	Standardized *Withania somnifera* extract	100	40
2	Starch	25	10
3	Talc	1	0.4
4	Magnesium stearate	1	0.4
5	Lactose	163	65.2

Table 2 : Formulation of Asparatab

S. No.	Ingredient	Weight of Tablet (300 mg)	Weight for 400 Tablets (gm)
1	Standardized *Asparagus racemosus* extract	100	40
2	Starch	25	10
3	Talc	1	0.4
4	Magnesium stearate	1	0.4
5	Lactose	163	65.2

Table 3 : Formulation of Centab

S. No.	Ingredient	Weight of Tablet (300 mg)	Weight for 400 Tablets (gm)
1	Standardized *Centella asiatica* extract	100	40
2	Starch	25	10
3	Talc	1	0.4
4	Magnesium stearate	1	0.4
5	Lactose	163	65.2

Table 4 : Formulation of Ascenwit

S. No.	Ingredient	Weight of Tablet (500 mg)	Weight for 400 Tablets (gm)
1	Standardized *Withania somnifera* extract	100	40
2	Standardized *Asparagus racemosus* extract	100	40
3	Standardized *Centella asiatica* extract	100	40
4	Starch	25	2.5
5	Talc	1	0.4

μg/ml, Centab showed IC_{50} value of 216.54 μg/ml (Table 6). In nitric oxide model Ascenwit exhibited IC_{50} value of 103.05 μg/ml, Withatab showed IC_{50} value of 195.84 μg/ml), Asparatab showed IC_{50} value of 299.04 μg/ml, Centab showed IC_{50} value of 217.95 μg/ml (Table 7). In ABTS model Ascenwit exhibited IC_{50} value of 99.96 μg/ml, Withatab showed IC_{50} value of 293.48 μg/ml, Asparatab showed IC_{50} value of 391.61 μg/ml, Centab showed IC_{50} value of 188.39 μg/ml (Table 8).

Discussion

With the passage of time and enhanced

Table 5 : Effect (%inhibition) of individual and combined tablet formulations on DPPH radical scavenging assay

Concentration (µg/ml)	%Inhibition			
	Withatab	Asparatab	Centab	Ascenwit
50	3.65	11.01	10	48.11
100	4.11	14.83	12.12	54.64
200	9.56	28.11	16.18	68.16
300	25.88	56.08	66.16	71.17
400	52.06	63.86	71.12	78.18
500	58.83	78.11	89.26	90.2
IC_{50} (µg/ml)	380.95	267.47	226.72	51.96

Table 6 : Effect (%inhibition) of individual and combined tablet formulations on Superoxide Radical Scavenging Assay

Concentration (µg/ml)	%Inhibition			
	Withatab	Asparatab	Centab	Ascenwit
50	10.08	12.03	14.03	50.11
100	12.73	14.12	15.26	58.26
200	24.09	28.17	46.18	62.13
300	50.07	56.16	58.88	67.18
400	59.16	61.18	65.28	74.17
500	65.57	67.27	70.17	81.27
IC_{50} (µg/ml)	299.58	267.09	216.54	49.89

Table 7 : Effect (%inhibition) of individual and combined tablet formulations on nitric oxide radical scavenging assay

Concentration (µg/ml)	%Inhibition			
	Withatab	Asparatab	Centab	Ascenwit
50	22.11	18.08	23.14	44.17
100	28.87	20.12	26.17	48.52
200	51.06	33.28	45.88	66.27
300	55.17	50.16	58.83	70.18
400	58.08	58.11	60.17	74.56
500	62.06	61	66.18	78.87
IC_{50} (µg/ml)	195.84	299.04	217.95	103.05

Table 8 : Effect (%inhibition) of individual and combined tablet formulations on ABTS radical scavenging assay

Concentration (µg/ml)	% Inhibition			
	Withatab	Asparatab	Centab	Ascenwit
50	22.06	9.1	14.15	46.17
100	26.06	12.88	21	50.22
200	43.72	18.86	53.08	55.27
300	51.11	34.14	58.17	58.97
400	59.24	51.07	63.19	68.17
500	62.22	56.65	65.14	76.28
IC_{50} (µg/ml)	293.48	391.61	188.39	99.56

knowledge, it was realized that combination of drugs gives better and faster relief; hence "Formulation" a concept of combining the herbs with similar therapeutic activity came in to existence. There is no doubt that most herbs exhibit their effects owing to a variety of constituents and the idea of synergy within and between them is also gaining acceptance.

From the above results it was clear that the mixture or formulation possess greater activity than individual components. The individual extracts of *Withania somnifera*, *Asparagus racemosus* and *Centella asiatica* contributed a greater extent to the antioxidant properties of Ascenwit compared to Withatab, Asparatab and Ascenwit. This contribution of individual extracts towards the overall potency is synergistic in mechanism.

Conclusion

In conclusion, *Withania somnifera*, *Asparagus racemosus* and *Centella asiatica* along with their formulations thereof, have a definite role to play in the health care system around the globe. Our *in vitro* antioxidant studies provide sound scientific footing to enhance confidence on the traditional claims of *Withania somnifera*, *Asparagus racemosus* and *Centella asiatica*. Combination of two or three herbs can make a good formulation for the specific indication and hence Ascenwit could become a novel antioxidant formula for many diseases associated with free radicals generation. However, more detailed pre-clinical and clinical evidences are required to establish its potency.

Acknowledgement

One of the authors Prateek Kumar Jain is thankful to Indian Council of Medical Research (ICMR), New Delhi and M.P. Council of Science and Technology (MPCOST), Bhopal, India for providing financial assistance.

References

Auddy B., Ferreira M., Blasina F., Tripathi P.C., Seal T. and Mukherjee B. (2003): Screening of antioxidant activity of three Indian medicinal plants traditionally used for the management of neurodegenerative diseases, *J. Ethnopharmacol*., **84**, 131-138.

Cai Y. Z., Sun M. and Corke H. (2003): Antioxidant activity of betalains from plants of the Amaranthaceae, *J. Agr. Food Chem*. **51**, 2288-2294.

Chandrasekar D., Madhusudhana K., Ramkishrna S. and Diwan P. (2006): Determination of DPPH free radical scavenging activity by reversed-

phase HPLC: A sensitive screening method for polyherbal formulations, *J Pharma. Biomed. Anal*., **40**, 460-464.

Halliwell B. (1994): Free radicals, antioxidants, and human disease: curiosity, cause, or consequence, Lancet., **344**, 721-724.

Mclarty J. W. (1997): Antioxidants and cancer: the epidemiologic evidence. In: Garewal, H. S. (Ed.), Antioxidants and Disease Prevention. CRC Press: New York, pp. 45-66.

Niwa Y. (1991): Effect of Maharishi 4 and Maharishi 5 on inflammatory mediators with special reference to their free radical scavenging effect, *J. Clin. Pract*, **1,** 23-27.

Poulson H E. and Loft S. (1998): Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Cancer Pr*., **7**, 9-16.

Richards R T. and Sharma H M. (1991): Free radicals in health and disease, *Indian J. Clin. Pract*., **2**, 15-26.

Shreejayan D. and Rao M. N. A. (1997): Nitric oxide scavenging by curcuminoids, *J. Pharm. Pharmacol*., **49**, 105-107.

Sun J., Chu Y. F., Wu X. Z. and Liu R. H. (2002): Antioxidant and antiproliferative activities of common fruits, *J. Agr. Food Chem*., **50**, 7449-7454.

Vani T., Rajini M., Sarkar, S. and Shishoo, C. J. (1997): Antioxidant properties of the Ayurvedic formulation-Triphala and its constituents, *Int. J. Pharm*., **35**, 313-317.

Yang C. S., Landau J. M., Huang M. T. and Newmark H. L. (2001): Inhibition of carcinogenesis by dietary polyphenolic compounds, *Annu. Rev. Nutr*., **21**, 381-406.

Yen G. C. and Chen H. Y. (1995): Antioxidant activity of various tea extracts in relation to their antimutagenicity, *J. Agr. Food Chem*., **43**, 27-32.

Zheng W. and Wang S.Y. (2001): Antioxidant activity and phenolic compounds in selected herbs, *J. Agr. Food Chem*.**, 49**, 5165-5170.