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In-vitro antioxidant activity of an Adaptogenic Homeopathic formulation

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ABSTRACT - The Homeopathic system of medicine relies mainly on plants and minerals as drug components. There are several herbo-mineral formulations available, which were claimed for their potential as tonic. One of such Homeopathic formulations containing adaptogenic plants, as ingredients, was prepared in the laboratory. The laboratory formulation was subjected to evaluation of *in vitro* antioxidant potential and compared with that of the marketed formulation. The formulations as well as the individual components exhibited antioxidant properties. The total phenolic content of the formulation and of the individual components were determined and correlated with the antioxidant activities. The present study showed that the Homeopathic medicines, administered even in much diluted form; exhibit significant *in-vitro* activities, which also justify the principle of Homeopathy Doctrine.
KEY WORDS: Adaptogenic, Homeopathic system of medicine, *In-vitro* Anti-oxidant activity, Tonic.

INTRODUCTION

Homeopathy is one of the alternative systems of medicine (1) having a well-documented pharmacopoeia. The monograph of pharmacopoeia includes generally the information on individual substances used as medicament. It has become common practice, now in homeopathic system, similar to modern system of medicine, of providing a multi component dosage forms. These dosage forms contain tinctures of herbal products and minerals either in combination or in singular form.

There is paradox that the medicinal power of substance increases as its quantity decreases - even to the point of being physically absent from the solution (2). *Hahnemann* believed that the process of dilution and succussion or trituration actually released a 'spirit-like' healing power that is particularly adapted to work on the equally spirit-like vital force in people (3).

The homeopathic system of medicine relies mainly on plant and mineral components as drugs. There are several homeopathic herbo mineral formulations available, which are used as tonic. Tonics are preparations, which mitigate the conditions of weakness or lack of tone within the entire organism, or in particular organ (4). Tonic effect thus, can be considered, as a part of the adaptogenic potential of the formulation. Adaptogenic potential of the drug may be attributed to its modulation effect on the functions of the various physiological systems of the body (5). The word Adaptogens was, first defined in the 1950 by *Lazarev,* as substances that normalize body functions, strengthen systems and functions compromised by stress and have a protective effect against a wide variety of environmental and emotional stress (6). Adaptogens are characterized by their anti-stress effects towards stresses of a non-infectious variety. Thus, the experiments were designed to access the adaptogenic properties of the selected tonic formulation and individual components from homeopathic system of medicine by determining their *in vitro* antioxidant properties. One such marketed formulation (7), was also evaluated in similar manner. Homeopathic formulation contain very diluted forms of plant mother tinctures, may be up to 10^{-3} X concentration power and some mother tinctures of minerals which is present in dilution of 10^{-6} X power in a preparation. These are prepared by using special method and in absolute alcohol, which is known as trituration and succussion in homeopathic formulary language. Phenolic compound present in the plant mother tincture have been considered to be responsible for antioxidant properties *in vitro* (8, 9). The total phenolic contents of the formulations as well as of the individual components, thus, were also determined. In the present study an attempt, has been made to a set certain parameters of identification and evaluation of mother tinctures of individual plant drugs as well as their formulation although in very diluted form, but shows significant activity supporting the

principle of Homeopathic medicine "As the concentration decreases the potency or activity of drug is increases", when subjected to evaluate certain parameters of evaluation and identification.

MATERIALS AND METHODS

Materials

The marketed homeopathic Alfalfa tonic selected for present study was labeled to contains diluted forms of Mother Tincture each of *Alfalfa (Medicago sativa)* (10), *Cinchona officinalis* (11), *Avena sativa* (12), *Hydrastis Canadensis* (13), *Withania Somnifera* (14),claimed with adaptogenic properties. The plant materials, of which tinctures were used in formulation, were individually procured from the local market. The samples of plant materials were identified by comparing them with herbarium specimen preserved in the Botany Department of M.S. University of Baroda. Tincture of Hydrastis was directly obtained from a local market. All reagents used, were of, pure grade, unless mentioned, otherwise in the description.

Preparation of Mother Tinctures

The Mother Tinctures of each of the procured drug sample, were prepared in the laboratory, using the given procedure, and then subjected to dilution in different grades as prescribed in Homeopathic Formulary (15, 16). Alfalfa mother tincture was prepared by using fresh plant leaves of *Medicago sativa*, cut into small pieces and crushed in mortar and pastle.. Afresh juice was obtained from the crushed leaves of Alfalfa by expression method using a new linen cloth. The juice obtained was weighed and equal quantity by weight of alcohol was added to it. The mixture was shaken vigorously for sometimes then allowed to stand in cool and dry place for eight days. The tincture was filtered, and stored in glass bottle provided with non-porous velvet cork. This tincture is termed as mother tincture in Formulary of Homeopathy. 2 minims of this mother tincture was added to 8 minim of dilute alcohol and then the mixture was given 10 downward stroke of equal strength (succession), which gave 1X potency preparation. All succeeding potencies may then be prepared by taking 1 minim of the preceding potency and 9 minim of dilute alcohol.

Cinchona tincture was prepared by using weighed quantity of fine powder of dried bark of *Cinchona officinalis* and five times of its weight alcohol was mixed in a glass jar. After mixing the whole mass was kept in cool and dark place for 15 days. The clear tincture was decanted; the residue was strained by new linen cloth and was added to the previously decanted tincture. The filtered tincture was stored in glass stoppered bottle with non-porous velvet cork. The succeeding potencies may be prepared similarly as stated for Alfalfa tincture.

Withania tincture was prepared by using fresh roots of *Withania somnifera* which were cut in to small pieces and crushed in mortar and pestle. Every three parts of weighed roots were treated with two parts by weight of alcohol and allowed to macerate for five days. The remaining procedure followed was same as mentioned for cinchona mother tincture.

Avena tincture was prepared using pulverized seeds of *Avena sativa*. A weighed quantity was taken and to it double the quantity by weight alcohol was added. The content was allowed to macerate for eight days, after this period the tincture is decanted, and filtered through a linen cloth and, stored , 6 minim of this mother tincture are mixed with 8 minim of dilute alcohol and the mixture was given 10 downward stroke of equal strength (succession) to give 1X potency preparation. All succeeding potencies may be prepared by taking 1 minim of proceeding potency and 9 minim of dilute alcohol.

Calcium Phosphate mother tincture was prepared by triturating with one part by weight of calcium phosphate with 99 parts by weight of sugar of milk (Lactose), which gave first triturate. The second trituration was prepared by taking one grain of first triturate with 99 grains of sugar of milk. The third triturate was prepared by triturating 1 minim of second triturate with 99 grains of sugar of milk. One grain of third triturate was dissolved in 50 minim of purified water and mixed with 50 minim of alcohol yielded 4^{th} potency. All succeeding potencies were prepared by mixing 1 minim of preceding potency and 9 minim of dilute alcohol and giving it 10 downward strokes. Similar methods were adopted to prepare Ferrous phosphate tincture, Sodium phosphate tincture, Potassium phosphate tincture.

Preparation of formulation

A liquid formulation was prepared in the laboratory based on the labeled contents of a marketed homeopathic herbal formulation. The ingredients were incorporated in the following manner. Each 5ml tonic contains – Alfalfa tincture of 2X potency 0.325 ml, *Cinchona officinalis* tincture of 3X potency 0.0075 ml, *Withania somnifera* tincture of 2X potency 0.0175 ml, *Avena sativa* tincture of 3X potency 0.0175 ml, *Hydrastis canadensis* tincture of 2X potency 0.0175 ml, acid phosphate tincture of 2X potency 0.0175 ml, Ferrous phosphate tincture of 6^{th} potency 0.25 ml,

Sodium phosphate tincture of 6th potency 0.025 ml, Magnesium phosphate tincture of 6th potency 0.025 ml, Potassium phosphate tincture of 6th potency 0.025 ml, Calcium phosphate tincture of 6th potency 0.025 ml. The laboratory formulation was prepared by mixing one after another, the tinctures of different potency of specified quantity of each ingredient and finally the volume was made up with distilled water. The formulation so obtained was then used for further investigation on following lines.

Preparation of sample for analysis

The tinctures of *Withania somnifera, Avena sativa, Cinchona officinalis, Hydrastis Canadensis, Medicago sativa (Alfalfa),* Laboratory preparation and Market preparation were dried to obtain solid mass at control temperature not to exceed 50° C first on a water bath and then subjected to vacuum drying. The solid mass was then dissolved in methanol to obtain a uniform concentration of 1 mg/ml. These samples were used for further studies in following manner.

Total Phenolic content

Total phenolic content was determined using Folin - Ciocalteau method. Each of the 100µl of samples of tinctures as well as laboratory and market preparation was taken in to 25ml volumetric flask, to which 10ml of water and 1.5ml of Folin Ciocalteau reagent were added. The mixture was then kept for 5 min. and to it 4ml of 20% w/v sodium carbonate solution was added the volume was made up to 25ml with double distilled water. The mixture was kept for 30 minute until blue color develops. The samples were then observed at 765 nm in UV- visible spectrometer Shimadzu, UV-1601, Japan. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure *(17-19)*.

The DPPH free radical scavenging activity, Super oxide free radical scavenging activity and Nitric oxide scavenging activity were calculated using the using following formula:

$$\% \text{ Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

The result of Total Phenolic content, DPPH free radical scavenging activity, Super oxide free radical scavenging activity, Nitric oxide scavenging activity were compared for Laboratory and Marketed preparation by using paired t-test.

DPPH free radical scavenging activity

4.3mg of DPPH (1, 1-Diphenyl –2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150µl DPPH solution was added to 3ml methanol and absorbance was taken immediately at 516nm for control reading Different volumes, of samples of mother tinctures of different drug as well as laboratory and market preparation, measuring 50µl, 60µl, 70µl, were taken and the volume was made uniformly to 150µl using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150µl DPPH was added. Absorbance was taken after 15 min. at 516nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value for each mother tinctures as well as laboratory and market preparation were calculated *(20,21)*.

Super Oxide free radical scavenging activity

100µl Riboflavin solution [20 µg], 200µl EDTA solution [12mM], 200µl methanol and 100µl NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and reaction mixture was diluted up to 3ml with phosphate buffer [50mM] The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5min.This is taken as control. Different volumes of 50µl, 60µl, 70µl of samples of each of mother tinctures of different drugs as well as laboratory and market preparation, were taken and diluted up to 100µl with methanol, to each of this, 100µl Riboflavin, 200µl EDTA, 200µl methanol and 100µl NBT was mixed in test tubes and further diluted up to 3ml with phosphate buffer. Absorbance was measured after illumination for 5min. at 590nm on UV visible spectrometer Shimadzu, UV-1601, Japan. Similar procedure is followed for other tinctures and laboratory and market preparation and adjusting the test sample volume 100 µl. IC_{50} value for each mother tinctures as well as laboratory and market preparation were calculated (22, 23)

Nitric Oxide scavenging activity (24-26)

50µl, 60µl, 70µl, of each of the samples of mother tinctures of different drugs as well as laboratory and market preparation were taken in separate tubes and the volume was uniformly made up to 150µl with methanol to each tube 2.0 ml , of sodium nitroprusside (10 mM) in phosphate buffer saline was added.

The solutions were incubated at room temperature for 150 minutes. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer Shimadzu, UV-1601, Japan. Curcumin was used as positive control IC_{50} value for each mother tinctures as well as laboratory and market preparation were calculated.

RESULTS

The test for total phenolic content was carried out on individual tinctures as well on laboratory preparation and on market formulation. It was observed that tincture of cinchona had highest phenolic content then rest of the tinctures. The phenolic content of marketed and laboratory preparation were quite comparable as showed in *Table-1*. In the DPPH Free radical scavenging activity, the withania tincture showed very potent activity then rest of the tinctures, while the market and laboratory preparation showed potent to moderate activity as mentioned in *Table-2*. The Super Oxide free radical scavenging activity was found highest in hydrastis tincture while rest of tinctures and market as well as laboratory preparation showed significant activity as in *Table-3*. Among the tinctures studied, nitric oxide scavenging activity was found only in *Withania* and *Hydrastis* while moderate activity was also observed in market and laboratory preparation as mentioned in *Table-4*. The result of Total Phenolic content, DPPH free radical scavenging activity, Super oxide free radical scavenging activity, Nitric oxide scavenging activity were compared for Laboratory and Marketed preparation by using paired t-test and results were found to be significant for DPPH free radical activity and Nitric oxide activity while results of Total Phenolic content and Super oxide free radical scavenging activity were found extremely significant.

Table-1: Total Phenolic content

Sample	Total Phenolic Content In µg/ml
Withania somnifera	416.10 ± 0.51
Avena sativa	422.47 ± 1.19
Cinchona officinalis	7464.8 ± 2.19
Hydrastis canadensis	*421.67 ± 2.21*
Medicago sativa (Alfalfa)	*651.03 ± 1.51*
Laboratory Preparation	587.67 ± 1.82
Market Preparation	605.80 ± 1.36

** The value is expressed as µg of Gallic acid equivalent / ml of sample*

** The regression values and correlation of regression of Gallic acid were*

$y = 0.0042x + 0.0187$ and $R^2 = 0.9916$

* *The Market preparation and laboratory were compared by using paired t-test and t value found was 68.265 and p value is 0.002 which was considered extremely significant*

Table-2: DPPH Free radical scavenging activity

Dose	IC_{50} in µg/ml	Regression equation	R^2
Ascorbic acid (Standard)	*18.53 ± 1.30*	*y = 1.786x – 0.009*	*0.9996*
Withania somnifera	10.87 ± 1. 17	y =1.1691x + 45.06	0.9923
Avena sativa	57.92 ± 2.22	y =2.6109x - 101.21	0.9991
Cinchona officinalis	12.19 ± 1.02	y =1.5364x + 31.27	0.9788
Hydrastis canadensis	*17.80 ± 1.91*	*y =0.7301x + 36.99*	*0.9981*
Medicago sativa (Alfalfa)	*63.30 ± 2.92*	*y =0.8794x - 5.67*	*0.9949*
Laboratory Preparation	64.45 ± 1.07	y =1.9151x – 73.62	0.9987
Market Preparation	61.32 ± 2.19	y = 0.6935x + 8.86	0.9975

* *The sign R^2 is correlation of Regression.*

* *The Market preparation and laboratory were compared by using paired t-test and t value found was 4.840 and p value is 0.0401which was considered significant.*

Table-3: Super Oxide free radical scavenging activity)

Dose	IC_{50} in µg/ml	Regression equation	R^2
Ascorbic acid (Standard)	18.16 ± 2.19	y = 1.345x + 1.23	0.9998
Withania somnifera	50.74 ± 2.37	y = 0.9854 – 0.0054	0.9976
Avena sativa	68.38 ± 1.96	y = 1.0809x – 19.592	0.9966
Cinchona officinalis	66.70 ± 0.57	y = 1.079x – 21.62	0.9955
Hydrastis canadensis	*28.63 ± 1.27*	*y = 1.19x + 15.93*	*0.9989*
Medicago sativa (Alfalfa)	*48.68 ± 2.37*	*y = 0.7529x + 18.61*	*0.9983*
Laboratory Preparation	61.29 ± 1.57	y = 1.2055x – 23.885	0.9976
Market Preparation	66.71 ± 1. 67	y = 0.7082x + 2.759	0.9993

** The sign R^2 is correlation of Regression.*

* *The Market preparation and laboratory were compared by using paired t-test and t value found was 93.877 and p value is 0.0001which was considered extremely significant.*

Table-4: Nitric Oxide scavenging activity

Dose	IC_{50} in µg/ml	Regression equation	R^2
Curcumin (Standard)	10.52 ± 1.72	y = 1.234x – 8.112	0.9996
Withania somnifera	48.97 ± 0.87	y = 0.9105x + 5.415	0.9976
Avena sativa	-	-	-
Cinchona officinalis	-	-	-
Hydrastis canadensis	*50.83 ± 1.84*	*y = 0.6331x + 17.817*	*0.9991*
Medicago sativa (Alfalfa)	-	-	-
Laboratory Preparation	69.45 ± 1.23	y = 0.5378x + 18.171	0.9989
Market Preparation	67.47 ± 2.16	y = 0.4626x +18.787	0.9994

** The sign R^2 is correlation of Regression.*

** The Market preparation and laboratory were compared by using paired t-test and t value found was 4.563 and p value is 0.0448 which was considered significant*

DISCUSSION

Traditional medicament play an important role in our day to day life in spite of overwhelming influence of modern medicine in treatment of various disorders like diabetes, viral infection, rheumatic disease, allergic condition, obesity, respiratory diseases, cardiovascular diseases, etc. Although number of poly herbal formulations are used in traditional system but only a few are accepted in modern medicine due to lack of accurate method for their standardization and evaluation.

Homeopathic dosage forms are widely used, containing tinctures of herbal products and minerals either in combination or in singularly. In general, scientifically acceptable methods for standardization of these preparations are not available. The present studies were planned to evolve certain parameters, and generate some data for evaluation and standardization of a selected poly herbal homeopathic adaptogenic tonic from the market. A similar product was prepared in the laboratory and both were subjected for investigation. The mother tinctures used in the preparation were found rich in phenolic content and so also the laboratory and market preparation, even though these contain diluted quantities their anti oxidant activity can therefore be correlated with phenolic content.

The DPPH scavenging activity, super oxide scavenging activity and nitric oxide scavenging activity exhibited by the samples were comparable with reference compound, ascorbic acid in first two and Curcumin for the third activities respectively. The IC_{50} value of *Withania tincture, Hydrastis mother tincture, and Cinchona mother tincture* shows higher antioxidant activity. The *Alfalfa mother tincture* and *Avena mother tincture* have moderate antioxidant activity. The laboratory preparation and market preparation show moderate activity even though they contain lower concentration of these tinctures, may be due to some synergic action which support the principle of Homeopathy. On the whole, the studies offer impetus to the need of evaluation of marketed preparation of Homeopathic system in order to generate confidence, both among the practitioners and patients.

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