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PRELIMINARY PHYTOCHEMICAL SCREENING AND INVITRO ANTI OXIDANT ACTIVITY OF HERBOMINERAL COMPOUND RVF – 8

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ABSTRACT

The formulation RVF - 8 is a mixture of eight drugs. All drugs used in this formulation are from different parts of plant sources. The mixture of different eight drugs was extracted with ethanol and evaluated for antioxidant activity. The *in-vitro* antioxidant activity of ethanolic extract has been investigated by 1, 1-diphenyl, 2-picryl–hydrazyl free radical (DPPH), hydrogen peroxide (H_2O_2), reducing power assay and nitric oxide scavenging methods. The ethanol extract has showing antioxidant activity. The results have been compared with the standard ascorbic acid.

KEYWORDS: Antioxidant activity, RVF-8, Free radicals, ethanolic extract.

INTRODUCTION

Oxidation is one of the most important free radical-producing processes in food, chemicals and even in living systems. Free radicals play an important role in food and chemical material degradation, contributing also to more than one hundred disorders in humans.[1,6] Highly reactive free radicals and oxygen species present in biological systems can oxidize nucleic acids, proteins and lipids, initiating degenerative diseases.[7,8] Antioxidants significantly delay or prevent the oxidation of easily oxidable substrates. Plants contain high concentrations of

numerous redox-active antioxidants, such as polyphenols, carotenoids, tocopherols, glutathione, ascorbic acid and enzymes with antioxidant activity, which fight against hazardous oxidative damage of plant cell components. Plantsourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytates and phytoestrogenes have been recognized as having the potential to reduce disease risk. The intake of food rich in *a*-tocopherols, *ß*-carotene and ascorbic acid has been associated with reduced oxidative-stress related diseases. Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl, thus inhibiting the oxidative mechanism that lead to degenerative diseases.[9,12]

These compounds have antioxidant, antimutagenic and anticarcinogenic activities and also free radical scavenging properties. It was therefore aimed to investigate its antioxidant activity by various *in vitro models.*

Showing the ingredients of RVF- 8 with their pharmacodynamic actions

Table no. I.

Sl. no.	Name of the drug	Latin name	Parts used	Chemical constituents	Pharmacological properties
1	Shataavari	*Asparagus racemosus*	Root	Steroidal saponins, asparagines, arginine	To increase milk secretion, general tonic
2	Aswagandha	*Withania somnifera*	Stem	Alkaloids, steroidal lactones.	Nontoxic, antitumoral.
3	Vidaarikanda	*Pueraria tuberosa*	Tuber	β-amyloid protein, isoflavanoids, pterocarpene.	Nootropic activity.
4	Krouncha beeja	*Mucuna Prurita Hook*	Seeds	mucanain, serotonin	Anti-depressant.
5	Citraphalaa/ Lingini	*Diplocyclos palmatus Jeff.*	Seeds	Alkaloids, triterpinoids, steroids.	Helps in conception & prevent miscarriage
6	Gokshura	*Tribulus terrestris*	Fruits	Steroidal saponins	Spermatorrhoea, gonorrhoea
7	Swarnamaksika (Tapya) bhasma	*CopperPyrite/Chacopyrit(CuFes$_2$)*		Bhasma(Ash/catalyst) containsFe$_2$O$_3$,Fes$_2$,CUS,Sio$_2$	
8	Purified Shilajith	*Black Bitumen*			Potent rejuvenator & anti-aging

Preparation of RVF- 8

The drugs no.1,2,3,4,5,6 mentioned in above table are taken in equal quantity and powdered, no. 7,8 of each drug in the quantity of 50 mg/ 1gram added and mixed together and kept in a glass container. After preparation, the blended powder was filled in the bottles, sealed and labeled. This packed sample was used for further studies.

MATERIALS AND METHODS

Materials

All chemicals and solvents were of analytical grade. *O*-Phenanthro -line, NEDD (Napthylethylene diamine dihydrochloride), Hydrogen peroxide, were purchased from Prince Trading Academy. Sulfanilamide was purchased from Symed Labs Jeedimetla and Trichloroaceticacid was purchased from Chemicals and Chemicals Hyderabad.

The other chemicals used were sodium nitroprusside, ferric chloride, potassium ferricyanide, methanol, monosodium dihydrogen phosphate, di-sodium hydrogen phosphate, potassium dihydrogen phosphate. Ascorbic acid was used as standard for whole study.

Preparation of extracts

First the powered drug was subjected to extraction. The extracts were prepared by using hot air percolation technique using soxhlet apparatus, a process of extraction of a drug with a solvent with several daily shakings. This method was based on the extraction of active constituents by simple hot air percolation using ethanol as solvent. 150g of the powdered material was placed inside a thimble supported by cotton pads which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools and drips back down into the chamber housing the solid material.

The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After 24 hrs, the ethanolic extract was filtered and the marc was repeated two more times with the same solvent for effective extraction. Extract was concentrated by open air drying. And the acquired extract was stored in a dessicator.[13,14]

A Schematic Representation of Extraction

150g of powder was percolated with 500ml ethanol as solvent for several times

Filtered, extract is concentrated by evaporation.

↓

Dried in desiccators.

↓

Resulting material was found to weigh as follows.

↓

Ethanol-5.6g.

DPPH free radical scavenging activity

Materials

- 2, 2- diphenyl-1-picrylhydrazyl radical (DPPH).
- Ethanol.
- Ascorbic acid.

Experimental procedure

1. A 0.06mM solution of DPPH was prepared .The initial absorbance of the DPPH in ethanol was measured at 517nm and did not change throughout the period of assay. A 0.5ml solution of the sample of different concentrations was added to 3.5ml of ethanolic DPPH solution. The change in absorbance at 517nm was measured at 30 min and free radical scavenging activity was calculated as inhibition using following equation. Percentage DPPH radical scavenging activity =1-(A_s/A_c) ×100, where A_S; Absorbance of the DPPH solution containing samples. A_C; absorbance of the control solution without sample but with DPPH the percentages of DPPH reduced were plotted against the samples. The experiment was also conducted using ascorbic acid as a reference antioxidant. The samples were analyzed in triplicates.[15,16]

2. Reducing power assay

Materials

- Ascorbic acid.
- Phosphate buffer p^H6.6.
- Potassium ferricyanide (1%w/v).
- Trichloroacetic acid(10%w/v).
- Ferric chloride (1g/1000ml).
- Different concentrations of extracts.

Experimental procedure

Standard

Different concentrations of Ascorbic acid (final concentration 25-800µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [K Fe (CN)] (1g/l), then mixture was incubated at 50o C for 20 3 6 minutes. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl (1g/l) and 3 absorbance measured at 700 nm in UV-Visible Spectrophotometer.[17] Phosphate buffer was used as blank solution.

Test

Different concentrations of plant extract solution (final concentration 25-800µg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, p^H 6.6) and 2.5 ml potassium ferricyanide [K Fe (CN)] (10g/l) and then mixture was incubated at 50°C for 20 minutes. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl (1g/l) and absorbance measured at 700 nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

3. Hydrogen peroxide scavenging activity

Materials

- Ethanolic extracts of poly herbal formulation.
- Phosphate buffer p^H 7.4.
- Hydrogen peroxide 40Mm.
- Ascorbic acid.

Experimental procedure

Firstly different concentrations of ethanolic extract was prepared in the following range (25, 75,100,200,400,600,800ug/ml). Also the standard concentrations of ascorbic acid were prepared as stated above.

To 1ml of the ethanolic extract and standard preparations, 0.6ml of 40mM hydrogen peroxide in phosphate buffer were added and incubated for 10 minutes at 25°C. The absorbances were

measured at 230 nm using blank distilled water for the control and phosphate buffer p^H 7.4 for the test and standard.[18] The percent scavenging activity was determined as follows:

% scavenging activity of H_2O_2 = A_0-A_T/$A_{0\ X\ 100}$

Where; A_0 is the absorbance of control, AT is the absorbance of test.

Preparation of dilutions

Stock solutions of ethanolic extract and standard of 1mg/ml was prepared i.e. 100mg of the extract and standard was dissolved in 100 ml of deionized water.

Different concentrations were prepared as follows:

100mg/100ml.
↓
1000µg/ml.
↓
0.1ml+0.9ml de ionized water.
↓
100µg/ml stock solution.

4. Nitric oxide scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological p^H interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction.

Materials

- Ascorbic acid.
- Sodium nitro prusside(5Mm).
- Griess reagent.
- Phosphate buffer p^H 7.

Experimental procedure

Standard

5ml of Ascorbic acid solutions in the concentration range (25-800µg/ml) were prepared with phosphate buffer p^H7.4. To the test tubes, 5ml of sodium nitro prusside solution (5Mm) was added. Then the resultant mixture was incubated at 25º for 2 hrs. To 0.9 ml of the above solution, 0.9 ml of Griess reagent was added and the absorbances were measured at 546nm.[19]

Test

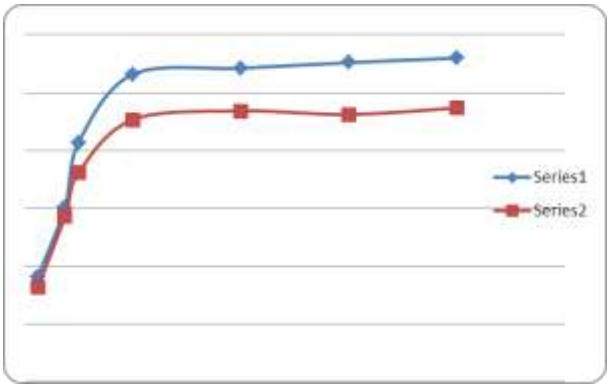
5ml of ethanolic extract (25-800µg/ml) was prepared with phosphate buffer ^{pH}7.4. To the test tubes, 5ml of sodium nitro prusside solution (5Mm) was added. Then the resultant mixture was incubated at 25º for 2 hrs. To 0.9ml of the above solution, 0.9ml of Griess reagent was added and the absorbances were measured at 546nm.

RESULTS

Table no: 3. DPPH activities.

CONC (µg/ml)	STANDARD PERCENT SCAVENGING	ETHANOLIC PERCENT SCAVENGING
25	18.21	16.28
75	30.18	28.62
100	41.32	36.12
200	53.12	45.31
400	54.21	46.82
600	55.16	46.21
800	55.95	47.33

Control value: 1. 44.



Graph for DPPH activity.

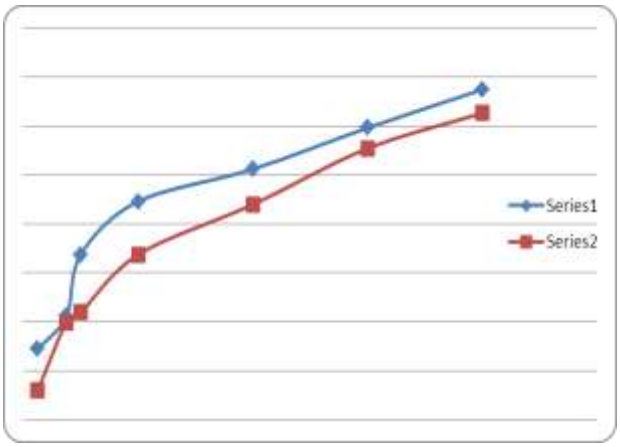
Series 1: standard**, Series 2**: ethanolic extract.

Graph was plotted between concentration and percent scavenging.

Table no. 4: Reducing power activity.

CONC (µg/ml)	STANDARD	PERCENT SCAVENGING	ETHANOLIC	PERCENT SCAVENGING
25	1.42	14.4	1.56	6.02
75	1.21	27.10	1.33	19.87
100	1.10	33.73	1.23	25.90
200	0.92	44.57	1.10	33.73
400	0.81	51.20	0.93	43.97
600	0.67	59.63	0.74	55.42
800	0.54	67.46	0.62	62.65

Control value: 1.66.



Graph for reducing power activity.

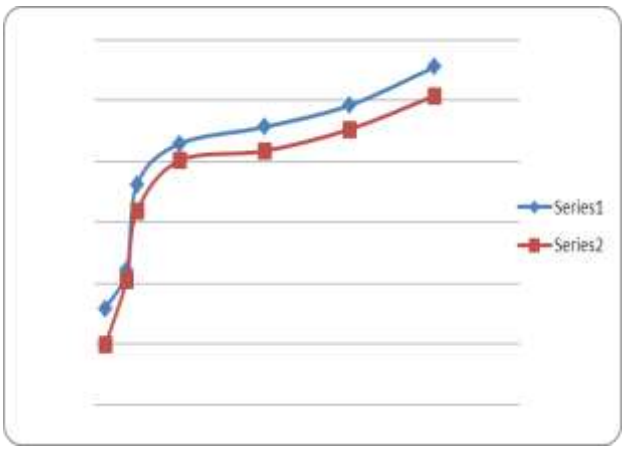
Series. 1: standard, **Series. 2**: ethanolic extract.

Graph was plotted between concentration and percent scavenging.

Table no. 5: Hydrogen peroxide scavenging activity.

CONC(µg/ml)	STANDARD	PERCENT SCAVENGING	ETHANOLIC	PERCENT SCAVENGING
25	2.14	15.74	2.29	9.84
75	1.98	22.04	2.02	20.47
100	1.62	36.22	1.73	31.88
200	1.45	42.91	1.52	40.15
400	1.38	45.66	1.48	41.73
600	1.29	49.21	1.39	45.27
800	1.13	55.51	1.25	50.78

Control value: 2.54.



Graph for hydrogen peroxide scavenging activity.

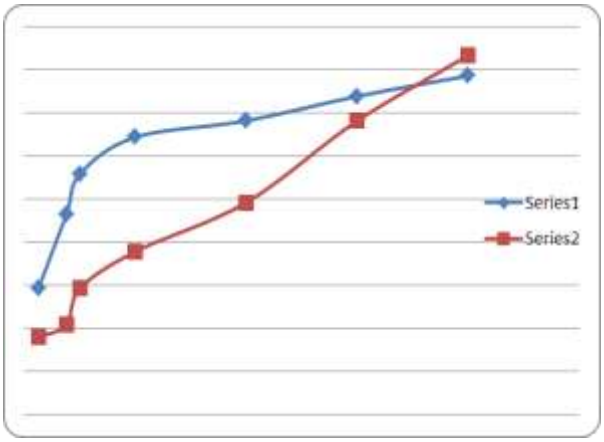
Series. 1: standard, **Series. 2:** ethanolic extract.

Graph was plotted between concentration Vs percent scavenging.

Table no. 6: Nitric oxide scavenging activity.

CONC (µg/ml)	STANDARD	PERCENT SCAVENGING	ETHANOLIC	PERCENT SCAVENGING
25	0.18	14.69	0.192	9.00
75	0.162	23.22	0.189	10.42
100	0.152	27.96	0.180	14.69
200	0.143	32.22	0.171	18.95
400	0.139	34.12	0.159	24.64
600	0.133	36.96	0.139	34.12
800	0.128	39.33	0.123	41.70

Control value: **0.211.**



Graph for Nitric oxide scavenging activity.

Series. 1: standard, **Series. 2**: ethanolic extract.

Graph was plotted between concentration and percent scavenging.

DISCUSSION

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by poly herbal ethanolic extracts.

Reducing power assay

Ethanolic extract had effective reducing power using the potassium ferricyanide reduction method when compared to the standard (Ascorbic acid) (Fig. 5). For the measurement of the reductive ability of ethanolic extract, the Fe3+–Fe2+ transformation was investigated in the

presence of ethanolic extract using the method of Oyaizu. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing power increased as the ethanolic extract concentration increased, indicating some compounds in poly herbal extract is both electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. The reducing capacity of a compound may serve as significant indicator of its potential antioxidant activity.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with $Fe^{2+,}$ and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.

It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by Poly herbal drug may at least partly result from its antioxidant and free radical scavenging activity.

DPPH free radical scavenging activity

Antiradical activity assay is based on the reduction of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting depolarization is stoichiometric with respect to the number of electrons captured.

CONCLUSION

The results obtained in the present study indicate that poly herbal ethanolic extracts exhibit significant free radical scavenging and antioxidant activity. The overall antioxidant activity might be attributed to its phytochemical constituents. The findings of the present study suggest that herbomineral drug could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases.

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