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SCREENING OF PHYTOCHEMICAL AND *IN-VITRO* ANTIOXIDANT PROPERTY OF A POLYHERBAL FORMULATION

P. Kavitha * and C. Sowmia

P. G. Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, Tamilnadu, India.

Keywords:

Polyherbal formulation, Synergistic effect, Total phenolics alkaloids, Anti-oxidants

Correspondence to Author:
P. Kavitha

P. G. Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, Tamilnadu, India.

Email: kavirajan83@yahoo.com

ABSTRACT: Herbal plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Thus, a proper scientific evidence or assessment has become the criteria for acceptance of herbal health claims. As such developing a polyherbal formulation will definitely produce synergistic effect as needed comparable to standard drugs that are available in market all over the world. The polyherbal formulation, which has a combination of medicinal herbs such as *Allium sativum, Trigonella foenum-graecum, Linum usitatissimum* was tested for its antioxidant activity, total phenolic and alkaloid contents *in-vitro.* The purpose of the present study was to investigate the *in-vitro* antioxidant, total phenolic and alkaloid content of polyherbal formulation and its application for treating life threatening diseases such as cancer, cardiac diseases, diabetes mellitus. In this paper we report the results of such studies in order to orient future investigations towards the finding of new, potent, safe and easily available food antioxidants.

INTRODUCTION: Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals [1]. Reactive oxygen species (ROS), which comprise free radicals like superoxide anion radicals (O_2.-), hydroxyl radicals (HO.) and non-free radical species such as H_2O_2 and singlet oxygen ($1\ O_2$), are forms of activated oxygen [2].

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It is increasingly being realized that many of today's diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of free radicals. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids, and deoxyribonucleic acid in healthy human cells and cause protein and deoxyribonucleic acid damage along with lipid peroxidation.

These changes contribute to formation of cancers, atherosclerosis, cardiovascular diseases, many other inflammatory diseases, and aging [3]. Synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used in processed food but they have side effects. Plant based antioxidants are now preferred to synthetic ones because of safety concerns [4]. There is an increasing interest in

natural antioxidants *e.g.* polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of diseases [5]. Different parts such as seeds, leaves and bark of stem and root known to contain substantial amounts of phytoconstituents such as phenolics, flavonoids, tannins having the ability to inhibit the free radicals that are excessively produced, hence can act as antioxidants [6]. The continued search among plant secondary metabolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic oxidants [7].

The purpose of the present study was to investigate the antioxidant properties, total phenolic and alkaloid contents of a polyherbal formulation containing *Allium sativum, Trigonella foenum-graecum, Linum usitatissimum* in combination. In this paper we report the results of such studies in order to orient future investigations towards the finding of new, potent and safe antioxidant foodstuffs.

MATERIALS AND METHODS:

Collection of plant materials:

The bulbs of *allium sativum*, seeds of *Trigonella foenum-graecum* and *Linum usitatissimum* were collected from super market, coimbatore. The specimens were identified by a taxonomist, Botanical Survey of India, Coimbatore.

Preparation of polyherbal formulation:

The bulbs and seeds were shade dried for 4-6 weeks and powdered finely in a mixture and sieved twice to obtain a fine powder. 100 gm of dried powder of each - *Allium sativum, Trigonella foenum-graecum, Linum usitatissimum* were separately extracted with Soxhlet extractor using 70 % ethanol till solvent was colourless. The extract was dried till constant weight was obtained. The yield was 21.16% for *Allium sativum,* 17.38% for *Trigonella foenum-graecum,* 15.90% for *Linum usitatissimum.* 25 mg of each extract was mixed together and henceforth labeled as Polyherbal formulation. This mixture of 25mg each of residue was dissolved in 10ml of ethanol, boiled in water bath for 5 minutes, cooled and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was used for evaluating antioxidant properties in various assays.

Phytochemical analysis:

The herbal preparation was subjected to preliminary phytochemical studies using standard procedures to detect the phytochemicals present. The polyherbal formulation was found to contain the important phytochemicals like Alkaloids, Flavanoids, Phenols etc., which proved to enhance the anti-oxidant potential.

Evaluation of *in vitro* antioxidant activity:

All chemicals and solvents used in the study were of analytical grade and are purchased from Hi-media chemicals.

Determination of total phenolics:

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark in the 50ml flask and left to react for 30 min for colour development. This was measured at 505 nm [8]. The analysis was performed in triplicate and the results were expressed as tannic acid equivalents.

Determination of total flavonoid content:

The flavonoid content was determined by the use of a slightly modified colorimetry method described previously by Zhishen *et al* [9]. A 0.5ml aliquot of appropriately (10mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

Determination of total alkaloid content:
Total alkaloid content was determined according to the method described by Fazel *et al* [10]. One ml of the sample solution (5mg/ml) was transferred to a separating funnel and then 5 ml of BCG solution (69.8 mg bromocresol green, 3 ml of 2N NaOH and 5 ml distilled water were mixed and raised to 1 L with distilled water) along with 5 ml of phosphate buffer (2 M sodiumphosphate adjusted to pH 4.7 with 0.2 M citric acid) was added. The mixture was shaken and the complex formed was extracted thrice with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The analysis was performed in triplicate and the results were expressed as atropine equivalent.

DPPH radical scavenging activity:
The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois [11]. The sample extracts at various concentrations (200 - 1000µg) was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as follows:

% DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Nitric oxide radical scavenging activity:
The nitric oxide scavenging activity of the sample was measured according to the method of Sreejayan and Rao [12]. 3ml of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (200 - 1000µg) of solvent extracts and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was read at 546 nm. Percentage radical scavenging activity of the sample was calculated as follows:

% NO radical scavenging activity = (control OD-sample OD / control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Superoxide radical scavenging activity:
Superoxide radicals were generated by a modified method of Beauchamp and Fridovich [13]. The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200 - 1000µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

% Superoxide radical scavenging activity = (control OD-sample OD / control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Hydroxyl radical scavenging activity:
The scavenging activity of the sample on hydroxyl radical was measured according to the method of Klein *et al* [14]. Different concentrations of the extract (200 - 1000µg) were added with 1ml of iron-EDTA solution (0.13% ferrous ammonium

sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated as follows:

%Hydroxyl radical scavenging activity = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Ferric reducing antioxidant power (FRAP):

The FRAP assay was used to estimate the reducing capacity of the sample, according to the method of Benzie and Strain [15]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, 2.5 ml of 20 mM FeCl3.6H2O and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900µl FRAP reagent was mixed with 90µl water and 10µl of the extract. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm.

RESULTS AND DISCUSSION:

The medicinal plants, which contain the high amount of polyphenols, are considered to be good source of natural antioxidant compounds and more often possess higher antioxidant potential than that of dietary fruits and vegetables. Consumption of these plant products certainly prevents the free radical mediated damage in the cell and therefore protects the body from several health problems. These antioxidant compounds can be used as natural antioxidant additives or nutritional supplements in the food products. As of natural origin, these antioxidants are much safe to use. Thus, much attention has been focused on the investigation of natural antioxidant compounds from plants, which can effectively scavenge ROS [18].

Qualitative analysis of selected species:

Literatures revealed that the selected four herbs *Allium sativum, Trigonella foenum-graecum, Linum usitatissimum* have antioxidant activity. Hence an attempt was made to formulate a polyherbal formulation, and to evaluate its *in vitro* antioxidant activity. Extraction and the phytochemical screening was done using 70% ethanol as the solvent. Phytochemical screening confirmed the presence of various phytoconstituents like alkaloids, phenols, carbohydrate, glycosides, flavanoids, tannins etc., and its showen in **Table 1.**

TABLE 1: QUALITATIVE ANALYSIS OF *ALLIUM SATIVUM, TRIGONELLA FOENUM-GRAECUM, LINUM USITATISSIMUM*

	Allium sativum	***Trigonella foenum-graecum***	***Linum usitatissimum***
Alkaloid	+	+	+
Flavanoids	+	+	+
Terpenoids	-	+	-
Glycosides	+	+	-
Tannins	-	+	-
Saponins	+	+	+
Cardiac glycosides	+	+	-
Steroids	+	+	+
Carbohydrates	+	-	+
Proteins	+	+	+

Quantitative analysis of selected species:

Chemicals present universally in all the plants can be classified as primary and secondary metabolites. Primary metabolites include proteins, amino acids, sugars, purines and pyrimidines of nucleic acids, chlorophylls etc., while secondary phytochemicals range from alkaloids to terpenoids and acetogenins to different phenols. These are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas [19]. During present investigation, the phytochemicals were quantitatively estimated in 3 common medicinal

plant and the results are shown in **Table 2** and **Fig. 1.**

TABLE 2: TOTAL PHENOLICS, FLAVONOID AND ALKALOID CONTENT

Sample	Total Phenolics (mg TAE/g sample)	Flavonoids (mg RE/g sample)	Total alkaloids (mg AE/g extract)
Polyherbal formulation (PHF)	19.32 ± 2.51	6.25 ± 0.12	25.07 ± 0.15

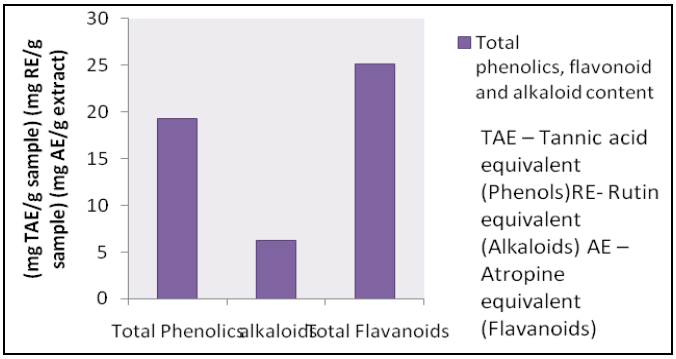

FIG. 1: TOTAL PHENOLICS, FLAVONOID AND ALKALOID CONTENT

DPPH free radical scavenging activity:

DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electrons become paired off and the solution loses color stoichiometrically depending on the number of electrons taken up [20]. Fig. 2 showed the DPPH scavenging effect increased with the increasing concentrations of polyherbal formulation as compared to standard ascorbic acid and IC_{50} value of the polyherbal formulation was observed as 159.03±14.86 µg/ml and IC_{50} value of standard ascorbic acid was 35.03 µg/mL, which indicates the DPPH scavenging as compared to ascorbic acid.

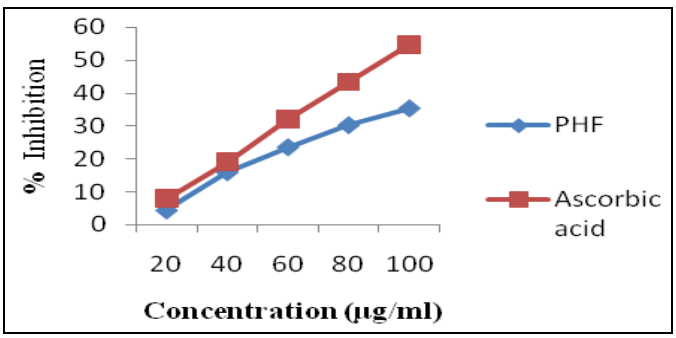

FIG. 2: DPPH RADICAL SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION

Ferric reducing antioxidant power:

The Ferric reducing antioxidant activity of the polyherbal formulation was given in **Fig. 3**. The 70% ethanolic extract of polyherbal formulation had a strong ferric reducing antioxidant power of 217.59± 0.39 mmol Fe II/ g of Sample and for standard ascorbic acid, it was found to be 356.2± 74.08 mmol Fe II/ g of Sample shows the similar ferric reducing property like that of Ascorbic acid.

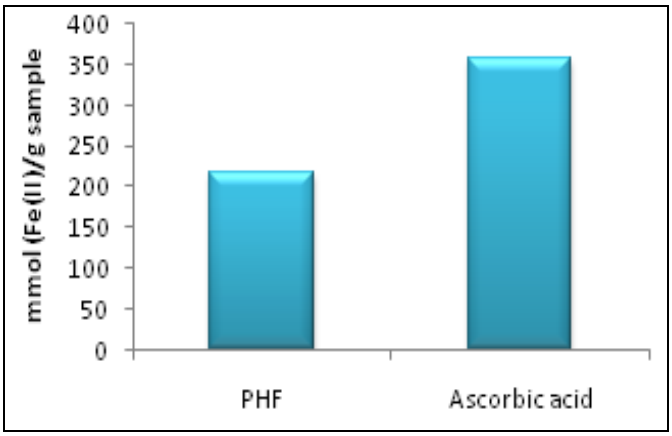

FIG. 3: FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

Superoxide scavenging activity:

Superoxides are produced from molecular oxygen due to oxidative enzymes of the body as well as by non-enzymatic reactions such as auto-oxidation by catecholamines [21]. The polyherbal formulation had a strong superoxide radical scavenging activity like that of the standard Ascorbic acid (**Fig.4**). The IC 50 value was found to be 117.99±4.18 for polyherbal formulation and for standard ascorbic acid, it was found to be 39.81 ± 0.22µg/ml.

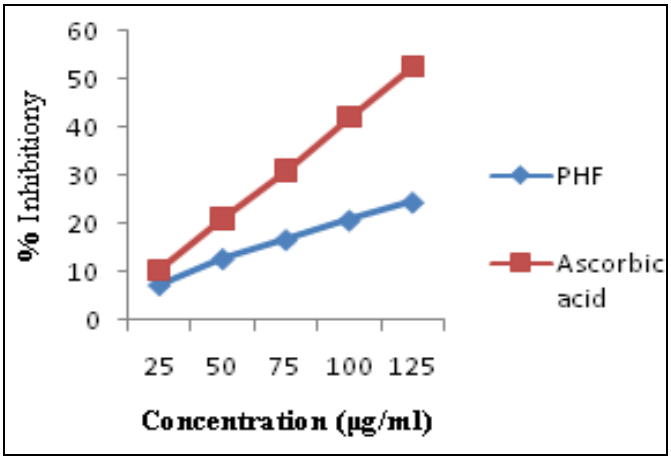

FIG. 4: SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION

Hydroxyl radical scavenging activity:

The hydroxyl radical scavenging activity is measured as the percentage of inhibition of Hydroxyl radicals generated in the fenton's mixture, by studying the competition between deoxyribose and the extract for hydrogen radicals generated from Fe^{3+} /ascorbate/ EDTA/ H_2O_2 systems. The hydroxyl radicals attack the deoxyribose which eventually results in TBARS formation [22]. The polyherbal formulation had a strong superoxide radical scavenging activity (**Fig.5**). The IC_{50} value was found to be 78.87±2.62 µg/ml for polyherbal formulation and for standard rutin, it was found to be 17.15 ±0.02µg/ml.

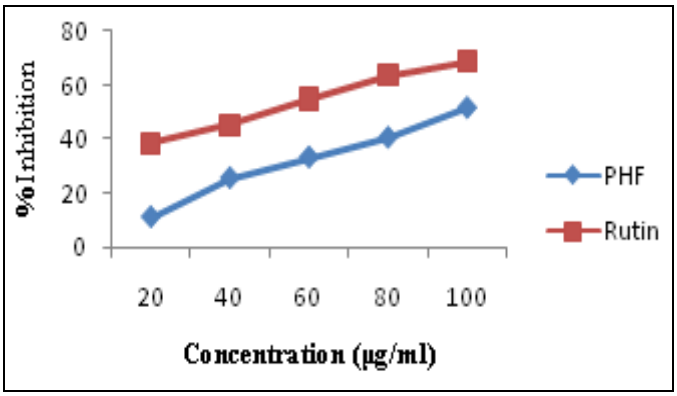

FIG. 5: HYDROXYL RADICAL SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION

Nitric oxide scavenging activity:

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological process [23]. The polyherbal formulation had a strong superoxide radical scavenging activity (**Fig. 6**). The IC 50 value was found to be 159.68±28.14 µg/ml for polyherbal formulation and for standard ascorbic acid, it was found to be 49.06 ± 0.18 µg/ml.

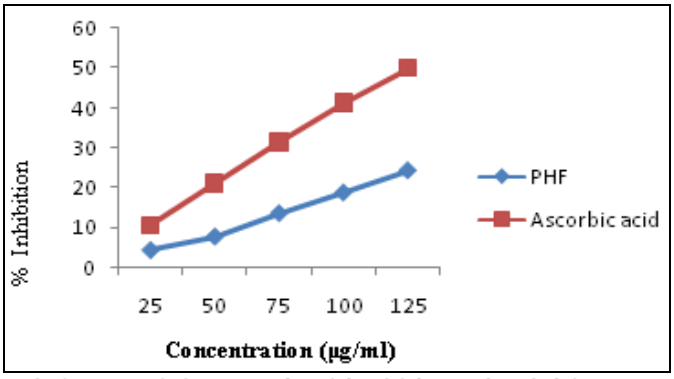

FIG. 6: NITRIC OXIDE RADICAL SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION

Previous studies suggested that the total polyphenolic content of the plant extracts will be positively correlated to the scavenging activities. The reducing potential of plant is mostly associated with the presence of reductones, which exert their mechanism of action by breaking the free radical chain by donating a hydrogen atom. The results obtained in the present studies may be attributed to several reasons viz, the inhibition of ferryl-perferyyl complex formation; scavenging of hydroxyl or superoxide radicals or by changing the ratio of Fe^{3+}/Fe^{2+}, reducing the rate of conversions of ferrous to ferric or by chelating of the iron itself. The phenolic compounds are one of the largest and most ubiquitous group of plant metabolites. They possess biological properties such as antiapopotosis, antiaging, anti carcinogen, anti inflammation, anti atherosclerosis, cardioprotection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferative activities [24].

CONCLUSION: The results of the present study showed that the ethanolic extract of polyherbal formulation contains phenols, flavonoids and alkaloids, which exhibited greater anti-oxidant activity. The high scavenging activity of the polyherbal formulation may be due to hydroxyl groups existing in the phenolic compounds, chemical structure that can provide the necessary component as radical scavenger. Free radicals are often generated as byproducts of biological reactions or from environmental factors. So the study shows that ethanolic extract of polyherbal formulation shows good anti-oxidant and free radical scavenging activity. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases like Cardio vascular diseases, Diabetes mellitus etc.,

CONFLICTS OF INTEREST: The authors have none to declare.

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