IN VITRO ANTIOXIDANT STUDY OF LIVOSYP: A POLYHERBAL FORMULATION

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Summary

Free radicals are highly reactive chemical species indicated in various diseases and ailments. In this study, the antioxidant activity of Livosyp, a polyherbal formulation, is evaluated by various antioxidant assays like total reducing power, DPPH free radical scavenging, hydroxyl free radical scavenging, hydrogen peroxide scavenging and nitric oxide scavenging including total phenol and flavonoid content. The various antioxidant activities were compared to standard antioxidants such as butylated hydroxyl toluene (BHT) and ascorbic acid. The results from the above studies indicate that Livosyp possesses potent antioxidant activity. The antioxidant activity is quantitatively equivalent to that of the standards used, i.e., BHT and ascorbic acid.

Key words: Free radical scavenging, antioxidant assay, Livosyp, Butylated HydroxyToluene, Ascorbic acid.

Introduction

A free radical is a chemical species that has an odd number of electrons, due to the presence of one or more unpaired electrons. These free radicals may be either oxygen derived or nitrogen derived. The oxygen derived species include superoxide, hydroxyl, hydroperoxyl, peroxyl and alkoxyl as free radicals; and hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen as non-radicals. Similarly, the nitrogen derived species include nitric oxide, nitrogen dioxide, peroxynitrite and dinitrogen trioxide. The production of free radicals (FR)/reactive oxygen species (ROS) is a naturally occurring intracellular process and a normal consequence of a variety of essential biochemical reactions. These free radicals, because of their relatively unstable nature, are highly reactive and damage number of biomolecules viz. lipids, proteins, enzymes, nucleic acids by oxidizing them.

The body is equipped with antioxidant defenses like reduced glutathione (GSH), glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) that provide protection against oxidative damages and help to maintain cellular homeostasis [1]. There are also various molecules which act like 'substrate suicide', or as an antioxidant shield. Among these molecules, some are lipophilic, such as tocopherols, carotenoids and ubiquinones [2]. Other hydrophilic molecules like ascorbic acid and uric acid are active in a hydrated environment. However, when the generation of FR/ROS far exceeds the levels of antioxidant defenses, oxidative damages can lead to the functional impairment in cells and even cause its death and the prevailing condition is termed as oxidative stress. Reactive oxygen species (ROS) are produced by all aerobic cells and are widely believed to play a pivotal role in aging as well as a number of degenerative diseases. Oxidative stress is implicated in several clinical conditions like aging [3], Alzheimer's disease [4], diabetes [5], hypertension, hepatic disorders [6], ischemic disorders, renal failure; and the evident pathologies in these disorders are either solely or partly attributed to the cytotoxic effect of the free radicals. Subsequently, the adjuvant antioxidant therapy has been proved to be useful for either correcting the disease or ameliorating the symptoms.

Livosyp is a polypharmaceutical herbal formulation containing aqueous extracts of 15 medicinal plants used for liver ailments. Livosyp contains: *Phyllanthus niruri* (Euphorbiaceae) [7], *Boerhaavia diffusa* (Nyctaginaceae) [8], *Eclipta alba* (Compositae) [9], *Berberis aristata* (Berberidaceae) [10], *Tinospora cordifolia* (Menispermaceae)[11], *Andrographis paniculata* (Acanthaceae) [12], *Picrorhiza kurroa* (Scrophulariaceae) [13] Russol *et al*., 2001), *Tephrosia purpurea* (Fabaceae)[14] , *Plumbago zeylanica* (Plumbaginaceae) [15], *Embelia ribes* (Myrsinaceae) [16], *Cichorium intybus* (Compositae) [17], *Solanum nigrum* (Solanaceae) [18], *Convolvulus turpethum* (Convolvulaceae), *Tecomella undulata* (Bignoniaceae) and *Fumaria vaillanti* (Fumariaceae). The present study is designed to investigate the free radical scavenging activity of Livosyp to generate scientific data as a proof of bioefficacy for finished herbal formulation.

Materials and Methods

Material

1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma), deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid, , ascorbic acid and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylene diamine tetra acetic acid (EDTA), aluminium nitrate, potassium acetate and Folin-Ciocalteu reagent were purchased from Qualigens Fine Chemicals (Mumbai, India). All the chemicals used including the solvents, were of analytical grade.

Total phenolic content

Total soluble phenolics present in the sample were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton [19] (1977) using pyrocatechol as a standard. Briefly, 1 ml of extract solution (1 mg/ml) in a volumetric flask is diluted with distilled water (46 ml). Folin-Ciocalteu reagent (1 ml) was added and

the contents of flask were mixed thoroughly. After 3 min, 3mL of Na_2CO_3 (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the extract is determined as microgram of pyrocatechol equivalents by using an equation that was obtained from standard pyrocatechol graph is given as:
Absorbance = 0.00246 µg pyrocatechol + 0.00325

Total flavonoid concentration

Flavonoid concentration was determined using the method described by Park *et al*., (1997) [20]. Extract solution (1 ml) was diluted with 4.3 ml of 80% aqueous ethanol and 0.1 ml of 10% aluminum nitrate and 0.1 ml of 1 M aqueous potassium acetate were added. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard.
Absorbance = 0.002108 µg quercetin – 0.01089

DPPH radical scavenging activity

The antioxidant activity of the samples, on the basis of the scavenging activity of the stable DPPH free radical, was determined by the method described by [21] Braca *et al*. (2001). One ml of various concentrations of the sample was added to 3ml of a 0.1 mmol/l methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as:
% inhibition = $(1- A_e/A_o) \times 100$,
where A_o is the absorbance without sample and A_e is absorbance with sample.
Ascorbic acid and butylated hydroxytoluene (BHT) were used as the positive control.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined according to the deoxyribose method of Siddhuraju and Becker (2007) [22]. To 1ml of appropriately diluted sample, 1ml phosphate buffer (20 mmol/l, pH 7.4) containing 0.1 mmol/l ferric chloride, 0.1 mmol/l EDTA, 2.8 mmol/l deoxyribose, 0.1 ml ascorbic acid (1 mmol/l) and 0.5 ml hydrogen peroxide (20 mmol/l) were added. Following incubation at 37°C for 90 min, 1ml of TCA (0.06 mol/l) and 0.3 ml of 0.2 mol/l TBA were added to the reaction mixture, which was then heated in a boiling water bath for 15 min. The absorbance of the pink color developed was measured at 532nm. The hydroxyl radical scavenging ratio was calculated by following formula:
% inhibition = $(1- A_e/A_o) \times 100$
where Ao is the absorbance without sample and Ae is absorbance with sample.
BHT and ascorbic acid were taken as positive control.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity was determined using the method of Ruch *et al.*, (1989) [23]. Sample with different concentrations was added to 3.4ml of 0.1M phosphate buffer (pH 7.4) and mixed with 600ml of 43mM solution of hydrogen peroxide. After 10 min, the absorbance at 230nm of the reaction mixture was recorded.

For each concentration, mixture without sample was used as control and mixture without hydrogen peroxide was used as blank and percentage inhibition was calculated.
% inhibition = (1- A_e/A_o) x 100,
where A_e is absorbance of sample and A_o is the absorbance of control.
BHT and ascorbic acid were taken as positive control.

Reducing power

The determination of reducing power was performed as described by Oyaizu (1986) [24]. One ml of the various concentrations of the sample solution was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). After the mixture was incubated at 50°C for 20 min, trichloroacetic acid (2.5 ml, 10%) was added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) after which the absorbance was measured at 700 nm. Increasing absorbance of the reaction mixture indicates increasing reducing power. BHT and ascorbic acid were taken as positive control.

Nitric oxide scavenging activity

Sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of sample and incubated at room temperature for 150 min. The same reaction mixture without the sample but the equivalent amount of methanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1- naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm [25].
The percent inhibition activity was calculated as
% inhibition = (1- A_e/A_o) x 100,
where A_o is the absorbance without sample and A_e is absorbance with sample.
Ascorbic acid and butylated hydroxytoluene (BHT) were used as the positive control.

Results and Discussion

The amount of phenolic components is calculated as pyrocatechol equivalents [19]. It is found to be 73.88 ± 0.23 µg mg^{-1} pyrocatechol equivalents. The total flavonoid concentration was measured as quercetin equivalents and is found to be 28.27 ± 0.28 µg mg^{-1} quercetin equivalents. It is well known that plant phenolic compounds and flavonoids are highly effective free radical scavengers. The Folin-Ciocalteu phenol method is actually not an antioxidant test but a replaceable assay for the quantity of oxidizable substance, i.e. phenolic compounds [26]. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent.

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH free radical scavenging activity of Livosyp is nearly similar to that of the standards used and is indicated in table 1. The model of scavenging the stable DPPH radical is widely used for relatively rapid evaluation of antioxidant activities. The essence of the DPPH method is that the antioxidants react with

the stable free radical, i.e. 1,1-diphenyl-2-picrylhydrazyl (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration. The antioxidant power is indicated by the degree of discoloration which could be determined by measuring of a decrease in the absorbance at 517 nm [27].

Hydroxyl radical is one the most deleterious and commonly formed free radicals. The scavenging activities of Livosyp, BHT and ascorbic acid on hydroxyl radical are quantitatively similar which is depicted in table 2. Both the DPPH free radical scavenging and hydroxyl radical scavenging activities of Livosyp are found to be concentration dependent. Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the reactive oxygen species (ROS) [28], which could be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron and cause the aging of human body and some diseases. They interact with the purine and pyrimidine bases of DNA as well as abstract hydrogen atoms from biological molecules (e.g. thiol compounds), leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecules [29]. Hydroxyl radical formation can occur in several ways; by far the most important mechanism *in vitro* is the Fenton reaction where a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide [30]. An *in vitro* Fenton type assay system ($Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$) that measures the generation of OH radicals and its subsequent effect on deoxyribose oxidation was used. The system involves autoxidation of an iron (II) – EDTA complex in aqueous solution to form O_2^-, which is rapidly dismutated to H_2O_2 at pH 7.4. The H_2O_2 further interacts with iron (II), to form OH radicals in the presence of ascorbic acid as a catalyst. Deoxyribose is degraded to malonaldehyde when exposed to $OH^{\bullet}$, which generates a pink compound with thiobarbituric acid, at low pH under heating. The effects are expressed as the percentage inhibition of the deoxyribose degradation to malonaldehyde.

Table 3 shows the comparative total reducing power of Livosyp, BHT and ascorbic acid which steadily increases with increase in concentration as indicated by the increasing absorbance values. The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity [31]. The exist of reductones are the keys of the reducing power, which exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom. In this assay, the reducing power was determined using a modified iron (III) to iron (II) reduction assay [32]. The presence of reductants in the solution causes the reduction of the Fe^{3+}/Ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power [33].

The nitric oxide scavenging activity of Livosyp is also quantitatively equivalent to that of the standards used and is indicated in the table 4. These activities are also found to increase with increase in concentration. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO^{3-} and NO^{2-} are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Nitric oxide is also implicated for

inflammation, cancer and other pathological conditions [34]. The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.

The hydrogen peroxide scavenging activity of livosyp is indicated in table 5. Hydrogen peroxide is not very reactive for itself, but it is toxic to cells because it may give rise to hydroxyl radical in the cells by Fenton and/or Haber-Weiss reactions [35]. Therefore, removal of H_2O_2 is very important for antioxidant defense.

Many of the herbs present in Livosyp are rich in polyphenols and flavonoids have been reported to have strong antioxidant activity. Antioxidant activity of herbs like *Phyllanthus niruri, Tinospora cordifolia*, *Embelia ribes*, *Solanum nigrum, Eclipta alba*, and *Plumbago zeylanica* present in the formulation has already been established and cited in the introduction of this article. When these pharmacologically active herbs are being used in conjunction, a synergistic additive effect is expected which could lead to high therapeutic efficiency [36, 37]. The results from the above studies indicate that Livosyp possesses significant antioxidant activity. The antioxidant activity is quantitatively equivalent to that of the standards used, BHT and ascorbic acid.

Table 1. DPPH free radical scavenging activity of Livosyp.

Concentration (µg/ml)	% inhibition		
	Livosyp	BHT	Ascorbic acid
25	78.72 ± 0.05	71.26 ± 0.03	76.76 ± 0.03
50	83.25 ± 0.03	77.47 ± 0.03	81.24 ± 0.02
75	85.29 ± 0.04	81.16 ± 0.03	84.34 ± 0.01
100	86.19 ± 0.03	83.28 ± 0.02	87.43 ± 0.01

Each value is expressed as mean ± SEM (n=4).One way ANOVA followed by Student-Newman-Keuls test was performed for the data obtained.

Table 2. Hydroxyl radical scavenging activity of Livosyp.

Concentration (µg/ml)	% inhibition		
	Livosyp	BHT	Ascorbic acid
25	74.25 ± 0.02	69.14 ± 0.02	73.25 ± 0.02
50	78.14 ± 0.02	73.15 ± 0.02	82.15 ± 0.03
75	82.34 ± 0.02	76.25 ± 0.03	85.35 ± 0.02
100	85.14 ± 0.01	81.13 ± 0.02	89.24 ± 0.02

Each value is expressed as mean ± SEM (n=4). One way ANOVA followed by Student-Newman-Keuls test was performed for the data obtained.

Table 3. Total reducing power of Livosyp.

Concentration (µg/ml)	Absorbance values at 700nm		
	Livosyp	BHT	Ascorbic acid
25	0.43 ± 0.006	0.33 ± 0.006	0.62 ± 0.005
50	0.73 ± 0.006	0.54 ± 0.008	0.83 ± 0.01
75	0.92 ± 0.008	0.82 ± 0.008	1.03 ± 0.006
100	1.14 ± 0.006	1.22 ± 0.008	1.32 ± 0.008

Each value is expressed as mean ± SEM. (n=4). One way ANOVA followed by Student-Newman-Keuls test was performed for the data obtained.

Table 4. Nitric oxide scavenging activity of Livosyp.

Concentration (µg/ml)	Absorbance values at 700nm		
	Livosyp	BHT	Ascorbic acid
50	32.28 ± 0.03	29.40 ± 0.02	25.63 ± 0.01
100	44.44 ± 0.02	40.29 ± 0.03	34.42 ± 0.03
150	59.29 ± 0.03	51.65 ± 0.02	43.76 ± 0.03
200	67.35 ± 0.04	62.12 ± 0.01	54.24 ± 0.02

Each value is expressed as mean ± SEM. (n=4). One way ANOVA followed by Student-Newman-Keuls test was performed for the data obtained.

Table 5. Hydrogen peroxide scavenging activity of Livosyp.

Concentration (µg/ml)	% inhibition		
	Livosyp	BHT	Ascorbic acid
5	26.20 ± 0.03	29.46 ± 0.03	27.33 ± 0.03
10	36.35 ± 0.02	38.76 ± 0.03	40.17 ± 0.03
15	42.16 ± 0.03	44.39 ± 0.02	47.47 ± 0.03
20	52.26 ± 0.03	53.18 ± 0.02	54.69 ± 0.02
25	65.43 ± 0.02	64.67 ± 0.02	66.75 ± 0.01
30	75.65 ± 0.03	77.29 ± 0.02	79.60 ± 0.02

Each value is expressed as mean ± SEM (n=6). One way ANOVA followed by Student-Newman-Keuls test was performed for the data obtained.

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