Available online on 15.08.2020 at http://ajprd.com

Asian Journal of Pharmaceutical Research and Development

Open Access to Pharmaceutical and Medical Research

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Research Article

In-vitro Antioxidant and Free Radical Scavenging Activity of A 50% Hydroethanolic Polyherbal Crude Extract

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A B S T R A C T

The multiple activities of the plant based medicinal preparations meant for varieties of inflammatory diseases offer enormous scope for combating the threat of diseases. The present study was designed to develop safer, effective and viable anti-inflammatory herbal combination to control oxidative stress related ailments as new alternatives to synthetic drugs. The synergistic potential of herbal combination of two medicinal plants was assessed through curative and preventive mode of treatment. Among the numerous traditional herbs, *Gymnea sylvestre* and *Urgenia Indica* is widely used for its traditional value. Free radicals induce numerous diseases by lipid peroxidation, and DNA damage. It has been reported that numerous plant extracts have antioxidant activities to scavenge free radicals. In the present study, the antioxidant properties of crude 50 % hydroethanolic polyherbal crude extract of *Gymnea sylvestre* and *Urgenia Indica* were studied in *in-vitro* models viz. radical scavenging activity by DPPH reduction Assay, Scavenging of SO, H_2O_2 and reducing power, DOT PLOT and FRAP assay. The extract was found to contain large amounts of phenolic compounds and flavonoids. 50% hydroethanolic polyherbal crude extract of *Gymnea sylvestre* and *Urgenia Indica* possessed significant antioxidant activity. These results suggest that synergistic activity of the crude extract has potential to develop a new functional dietary agent to treat chronic metabolic diseases, such as diabetes , hyperlipidemia and various inflammatory diseases.

Keywords: *Gymnea sylvestre*, *Urgenia Indica*, antioxidant capacity, scavenging activity, Reducing power and DOT plot.

A R T I C L E I N F O: Received 04 -2020; Review Completed 05 July 2020; Accepted 18 July 2020; Available online 15 August. 2020

Cite this article as:

Sudhakar M*,Perumal AV, Rassappan R, Sambamurthy S, Kumar SS, Sundar S, Viswanathan V , Krishnan YL, Asian Journal of Pharmaceutical Research and Development. 2020; 8(4):30-36. DOI: http://dx.doi.org/10.22270/ajprd.v8i4.771

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INTRODUCTION

Free radicals are chemical species which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Living cells generate free radicals and other reactive oxygen species (ROS) by-products as a result of physiological and biochemical processes. In recent years, focus on plant research has increased all over the world. Collected evidences showed immense potential of medicinal plants used in various traditional systems, for their biological activities and antioxidant principles[1]. Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defence system against disease or more accurately, to protect against disease. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more[2]. Historically, the Ayurvedic literature "*Sarangdhar Samhita"* dated centuries in the past in 1300 A.D. has highlighted the inspiration of polyherbalism in this ancient medical system[3]. In the traditional system of Indian medicine, plant formulations and combined extracts of plants are chosen rather than individual ones. It is known that Ayurvedic herbs are prepared in a number of dosage forms, in which mostly all of them are polyherbal formulation[4].This phenomenon of herb-herb interaction is known as synergism. Certain pharmacological movements of the active constituents of herbals are significant only when patented by that of alternative plants, but not evident when used alone[5].

Numerous polyherbal formulations, which are combination of different herbal extracts/fractions, are used for the treatment of various 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[6].

G. sylvestre is an indigenous medicinal herb belonging to the class dicotyledonous of the family Asclepiadaceae. The plant is a good quality of many bioactive compounds[7]. It has deep roots in history, being one of the major botanical used in Ayurvedic system of medicine to treat conditions ranging from diabetes, malaria, to snake bite [8].

Urginea indica is rare, endangered and threatened Indian medicinal plant belongs to *Liliaceae* family, commonly called as sea onion[9]. It is a perennial plant having fibrous roots from the bottom of a large, tunicated and globular bulb. There are two varieties of *U. indica*, which differ in the color of bulb scales, have been reported to possess medicinal properties[10]. Nearly all parts of the plants are reported to have therapeutic value; recently, bioactive compounds of bulbs have received much attention due to their anti-cancer properties[11].

However, no data are available in the literature on the antioxidant activity and free radical scavenging activity of the polyherbal crude extract on these two significant medicinal plants. Therefore we undertook the present investigation to examine their synergistic potential on in-vitro models.

MATERIALS AND METHODS:

Plant Collection and Authentification:

The plant sample was collected in and around Coimbatore and is authenticated in botanical survey of India (BS1) in TNAU, Coimbatore. A voucher specimen (No: Bs1/SRC/5/23/2012/Tech44) has been deposited at the herbarium. The parts such as bulb and fruit are used for the study.

Preparation of A 50% Hydroethanolic Polyherbal Crude:

Each one gm of a polyherbal crude contains equal amount of *Gymnema Sylvestre*a and *Urgenia Indica*.20gm of the dried power of each plant was taken and cold macerated with 50% hydro ethanolic solvent with occasional. Stirred for 3 days. After 3 days the suspension was filled through a fine muslin cloth and filtrate was evaporated to dryness, until the solvent gets evaporated. The yield concentrated crude extract is called as polyherbal crude extract (PH) and stored in an air tight container or conical flask and used for further analysis.

Qualitative analysis of Phytochemicals

The qualitative phytochemical screening conducted on the crude extract showed the presence of a wide class of bioactive compounds, including alkaloid, terpenoids, phenols and tannins, reducing sugar, protein, steroid, anthocyanin, coumarin, leucoanthocyanin and glycosides.

Quantification of Bioactive Compounds:

Determination of total phenolic content (TPC)

The total phenolic content in the methanolic extract was determined with Folin-caltaeu method[12]. For this 1000 µg/ml gallic acid stock solution was prepared by dissolving gallic acid in methanol. 1 ml of gallic acid solution was mixed with 5.0 ml of 50 % Folin-ciocalteu reagent. After 5 min, 4.0 ml of 7.5 % Sodium Carbonate (solution was added to the mixture, was shaken and then incubated at room temperature for 30 min in dark. The absorbance of all samples was measured at 765nm spectrometrically. Gallic acid was used to prepare a standard curve by preparing 1 ml aliquots of 1.0, 2.5, 5.0, 10, 15, 20, 25, 50, 100 µg/ml of gallic acid solutions (y = 0.008x+0.018; r=0.992, where 'y'represents the absorbance, and 'x', the concentration). The result was expressed in milligrams, Gallic acid equivalents per gram of extract (mg GAE/g extract)

Total phenolic compound extract was determined by applying the following equation: C= C×V/M

Where; C=Total content of phenolic compound in mg/g, in GAE (Gallic acid equivalent),

C= Concentration of Gallic acid established from the clibration curve in mg/ml,

V= Volume of extract in ml,

m= Weight of plant extract in gm.

Determination of total flavonoid content

For estimation of total flavonoid content, aluminium chloride method was used[13]. In this method quercetin was used as standard and flavonoid content were measured as quercetin equivalent. For this purpose, the calibration curve of quercetin was drawn. 1ml aliquots of quercetin (20, 40, 60, 80, 100 µg/ml) was taken into 10 ml volumetric flask, containing 4 ml of distill water, 0.3 ml of 5 % NaNO added to the flask. After 5 minutes, 0.3 of 10 % AlCl3 was added and volume was made upto 10ml with distilled water and same process was done with the plant extract. The absorbance was noted at 510 nm using UV-visible spectrometer.

Determination of total alkoloids content

For estimation of alkaloids, 5 g plant sample was mixed with 20 ml, 10 % acetic acid in ethanol, incubated for 4 h and filtered. The filtrate was kept on water bath to make it concentrated or to make its volume 1/4th the original volume. To this, drop by drop concentrated ammonium hydroxide was added to precipitate alkaloid. This solution was left to settle and the precipitate was collected in a filter paper. The collected precipitate was washed with dilute ammonium hydroxide solution and dried in oven at 40°C, until a constant weight was obtained. Then alkaloid precipitate was calculated in mg/g of the dried plant material[14].

In vitro antioxidant assays

DPPH radicals scavenging activity

The free radical scavenging capacity of the crude extract was determined by using DPPH assay[15].DPPH solution (0.004%, w/v) was prepared in methanol. Stock solution (1 mg/ml) of methanol extract of plant and standard ascorbic acid (0.05 g/ml) were prepared using methanol. Various concentrations (10-500 µg/ml) of the plant extract and ascorbic acid were taken in a test tube and 1ml freshly prepared DPPH solution was added, the test tubes were protected from light by covering with aluminium foil. The

final volume in each test tube was made to 2 ml with methanol and incubated in dark for 30 mins at room temperature. After incubation the absorbance was read at 517 nm using a spectrophotometer. The control sample was prepared to contain the same volume of methanol and DPPH without any extract and reference ascorbic acid. Methanol was served as blank.

The radical scavenging was calculated by the following formula;

% Inhibition = [(Abs of control –Abs of sample)/ Abs of control] ×100

Superoxide anion radical scavenging activity[16]

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) under aerobic condition. Tris HCl buffer (3ml, 16 mM, pH 8.0) containing 1 ml NBT (50 µM) solution, 1 ml NADH (78 µM) solution and a sample solution of extract (10-500µg/ml) in distilled water mixed. The reaction was started when 1 ml of PMS solution (10 µM) was added to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was read at 560 nm against the corresponding blank samples. Ascorbic acid was used as a standard. The decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Hydrogen peroxide radical scavenging activity

The capability of the extract to scavenge hydrogen peroxide (H_2O_2) was estimated according to the method of Nabavi[17]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer, pH 7.4. The concentration of hydrogen peroxide was determined by absorption at 230 nm using a UV- visible spectrophotometer. The extracts (10-500µg/ml) in distilled water were added to a hydrogen peroxide solution at 230 nm was determined after 10 mins against the blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as a standard.

Reducing power assay

FRAP assay

The FRAP assay was performed[18]. FRAP reagent was prepared and kept at 35ºC for 5-10 min prior to use. In a 96 well plate, 175 µl of FRAP was added and the plate was pre read at 593nm. To each well, 25µl of sample solution was added and contents were mixed well using a pipette. Absorbance was measured at 593nm at 4, 15, 30 and 60 min, using methanol as blank. Change in absorbance at different time intervals was then translated in to a FRAP value using a standard curve.

DOT Plot assay

The DOT plot assay was performed[19]. An aliquot (10µl) of the different concentrations (100, 500, and 1000µg) of extract was carefully loaded onto a10 cm x 5 cm TLC sheet (silica gel 60 F254; Merck) and allowed to dry for 3 minutes. The plate was immersed in DPPH solution for 5 minutes. The change on the colour of DPPH solution from purple to yellow indicates the antioxidant potential of the plant

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening for the Qualitative Analysis:

The phytochemical compounds of 50% hydroethanolic polyherbal crude extract were qualitatively analysed and the results are revealed in Table1. The powder form of the polyherbal formula was subjected to extraction with hydro ethanol for 24hrs in the order of increasing the polarity of solvents was used for the phytochemical screening.

The phytochemical screening of the present study has revealed the presence of alkaloids, flavonoids, saponins, tannins, glycosides, phenols and steroids. Among the various extracts the hydro ethanol extract shows more positivity to maximum phytochemical constituents. Since, the yield of bioactive metabolites in these extracts also varies considerably with the solvent of extract. It is plausible that the hydro ethanolic extract generally more potent than the aqueous extract and ethanol extract, probably due to the active principles present in the plants dissolve more readily in and were better extracted by a less polar solvent and in water[20].

The two medicinal plants which are present in the polyherbal crude extract has been already reported individually in different extracts to contain the compounds like alkaloids, flavonoids, glycosides, lignins, phenols, tannins, sterols, thiols, saponins, fats, oils, quinines and coumarines (21,22,23,24).These constituents may be responsible for the medicinal activity as well as exhibiting physiological activity[25].

Henceforth, these primer screening tests might be helpful in the recognition of bioactive standards and in this manner may prompt the medication revelation and improvement. Further these tests facilitate their quantitative estimation and qualitative separation with pharmacologically important active chemical constituents present in the 50% hydroethanolic polyherbal crude extract.

Table 1: Phytochemical investigation of the formulation

S.No	Phytochemical Test	Hydro Ethanolic Poly Herbal Crude Extract
1	Reducing Sugar	+
2	Alkaloids	+
3	Carbohydrates	+
4	Glycosides	+
5	Tannins	+
6	Flavonoids	+
7	Steroids	-
8	Saponins	+

Quantitative Phyto Chemical Analysis of 50% Hydroethanolic Polyherbal Crude Extracts:

Some molecules produced during primary metabolism can participate in stress response and provide plant Secondary metabolism is key to plant adaptation to stress. However, defence properties[26]. The levels of flavonoids, alkaloids and phenols from 50% hydroethanolic polyherbal crude extract have been shown in Figure 1.

FLAVONOIDS:

Flavonoids are the phenolic substances which are the biggest gathering of phenols. They generally occur as a C6-C3unit linked to an aromatic ring. They are other plant constituents with antibacterial and antifungal properties [27]. The flavonoid content of the polyherbal crude extract was found to be 100 mg/g.

Phenols:

Phenols are the most widespread auxiliary metabolites in the plant kingdom. The differing gathering of mixes has gotten a lot of consideration as potential characteristic cell reinforcements, as far as their capacity to go about as both productive radical foragers and metal chelators. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[28]. In the present study, the total phenolic content was found to be 50 mg/g.

ALKALOIDS:

The alkaloid content of the crude extract was found to be as shown 10 mg/g. Alkaloids which are one of the largest groups of phytochemical in plants having beneficial effects on humans and this has led to the development of powerful pain killer modifications[29]. Also studies have showed that alkaloid is capable of reducing headaches associated with hypertension [30].

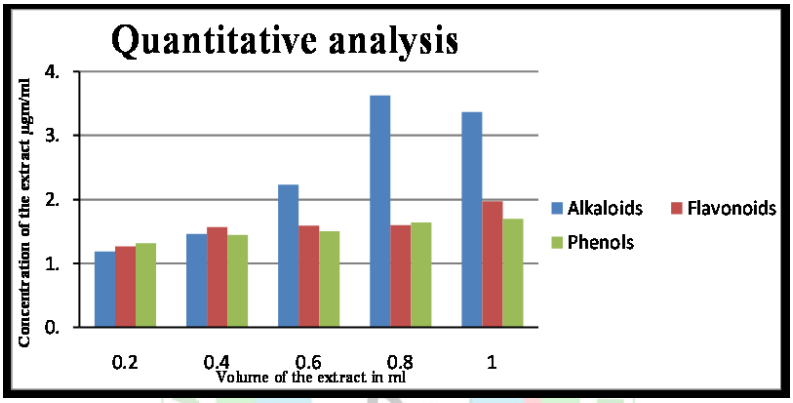

Figure: 1 Quantitative analysis of a Polyherbal crude extract

***In Vitro* Antioxidant and Free Radical Scavenging Activities of a Hydroethanolic**

Polyherbalcrudeextract:

Reactive oxygen species (ROS) including super oxide anion, hydroxyl radicals, DPPH and other free radicals are considered to be potent damaging chemical species formed in day to day life. Besides their deleterious effect, they also possess beneficial activities to living systems. Almost all the herbal based drugs possessing free radical scavenging potential have gained consistent importance in the treatment of chronic diseases[31].

***In vitro* Radical Scavengining Assays:**

The results of *in-vitro* radical scavenging (O_2^-, H_2O_2, DPPH) of 50% hydroethanolic polyherbal crude extract was shown in the Table 2.

Assay of Superoxide Radical Scavenging Assay:

Superoxide radical is not only one of the strongest reactive oxygen species among the generated free radicals, but also a precursor to other active free radicals such as hydrogen peroxide, hydroxyl radical and singlet oxygen which play an important role in the oxidative damage and there by inducing tissue damage[32,33,34].

The result of superoxide anion radical scavenging activity of 50% hydroethanolic polyherbal crude extract was shown in Table 2.

Superoxide anion reduces the yellow dye (NBT^{2+}) top reduce the blue formation which is measured at 560nm.The 50% hydroethanolicpolyherbal crude extract exhibited maximum superoxide radical scavenging activity at 57.18%.

In the present study, 50% hydroethanolicpolyherbal crude extract is examined for its ability to act as scavenging agent. This might be due to the antioxidant properties of some flavonoids, which are effective mainly through scavenging of superoxide scavenging radical. Free hydroxyl radical formed in the crude extract have the ability to degrade 2-deoxy ribose into fragments[35].

Hence, high inhibitory effects of the 50% hydroethanolic polyherbal crude extract on the superoxide anion formation showed here is possibly rendered them as promising antioxidants.

Assay of Hydrogen Peroxide Scavenging Activity:

Hydrogen peroxide is a powerless oxidizing operator and can inactivate not many catalysts legitimately, typically by oxidation of basic thiol gatherings. Hydrogen peroxide can cross cell film quickly and once inside the cell, hydrogen peroxide can most likely respond with Fe^{2+} and perhaps Cu^{2+} particles to frame hydroxyl radicals and this might be the birthplace of a large number of its harmful impacts. It is therefore biologically beneficial for cells to control the accumulated hydrogen peroxide [36].

The results given in the table 2 for the 50% hydroethanolic polyherbal crude extract has demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner.

The 50% hydroethanolic polyherbal crude extract exerted at the inhibition of 26.20%. However, the standard ascorbate was found to possess 92.84% scavenging activity which was higher than crude extract.

Scavenging of hydrogen peroxide may be attributed to the phenol compound present in the 50% hydroethanolic polyherbal crude extract, which donate electron the hydrogen peroxide thus reducing it to water[37, 38]. The oxidative activity of flavonoids comprised of an underlying huge disintegration impact on hydrogen peroxide followed by an ensuing period of extremely moderate deterioration[39].

Assay of DPPH Radical Scavenging Activity:

DPPH is as table nitrogen centered free radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents, and then losing colour stochiometrically with the number of electrons consumed[40]. DPPH assay has been carried out to measure antioxidants in complex and biological systems since it is easy and convenient[41].

Table 2: Scavenging activity of a 50% hydroethanolic polyherbal crude extract

S.NO	Concentration of The Ph Extract	Percentage of Scavenging Activity%					
		DPPH	Ascorbic Acid	Hydrogen Peroxide	Ascorbic Acid	Superoxide	Ascorbic Acid
1	100	36.32	36.70	14.21	40.03	22.16	23.03
2	200	42.86	44.68	20.50	47.77	34.13	35.48
3	300	49.29	50.70	22.05	57.83	40.01	40.91
4	400	52.28	53.35	25.53	64.31	53.08	53.83
5	500	54.93	60.81	26.20	92.84	57.18	58.89

Table 2 revealed the 50% hydro ethanoli polyherbal crude extract in the DPPH radical scavenging activity. The 50% hydroethanolic polyherbal crud extract were showed a maximum scavenging potential (54.93% and 60.81%) which was comparable with ascorbic acid. This result indicated that the plant was potently active and 50% hydroethanolic polyherbal crude extract did contain compounds that could be capable of donating hydrogen to a free radical in order to remove the odd electron which is responsible for the radical reactivity[42].

Reducing Power Assay:

Ferric Reducing Antioxidant Power (FRAP) assay:

FRAP assay is to determine the efficiency of antioxidant compounds in plants to compete with the FRAP reagent and reduce the ferric ion to the ferrous state. Antioxidant compounds that are able to function in this approach are categorized as secondary antioxidants where they suppress the radical formation and prevent oxidative damage [43].

The results obtained from FRAP values at different time intervals showed 104µm TE/mg at 4th min steadily followed of the increasing trend to 107µm TE/mg at 30th min further reaching at 109µm TE/mg at 60th min shown in the Figure 2.

The 50% hydroethanolic polyherbal crude extract has the ability to bring about the reduction of ferric ions in a time dependent manner. The reducing power of crude extract is being directly correlated with its antioxidant activity and it is based on the presence of reductant which exerts the antioxidant activity by breaking the free radical chain and donating a hydrogen atom[44, 45].

Similar to these results, [46]had reported on the antioxidant and free radical scavenging activity of *Triphala* determined by using different *in-vitro* models. This proves that there was a synergistic enhancement in the ferric reducing ability in the 50% hydroethanolic polyherbal crude extract.

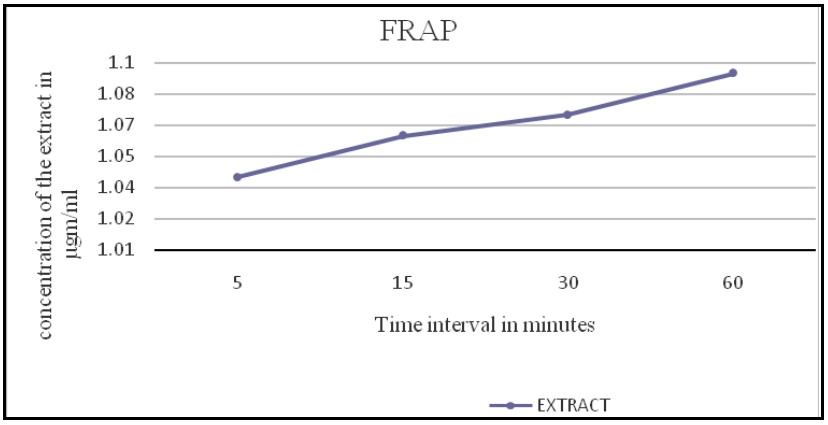

Figure: 2 Assay of FRAP in the hydroethanolicpolyherbal crude extract

DOT-PLOT Assay:

DPPH radicals are widely adapted to evaluate the scavenging potential of natural antioxidant. Figure 1 revealed the TLC based qualitative DPPH spray of the50% hydroethanolic polyherbal crude extract at six different concentrations (100,200,300,400,500,1000mg/ml).

The 50% hydroethanolic polyherbal crude extract showed prominent free radical scavenging properties indicated by

the presence of a yellowish green spot on a white background on the TLC plate with maximum zone at 400mg/ml. It is based on the inhibition of the accumulation of oxidized products, since the generation of free radicals is inhibited by the addition of antioxidants and the end point by scavenging the free radical[45].

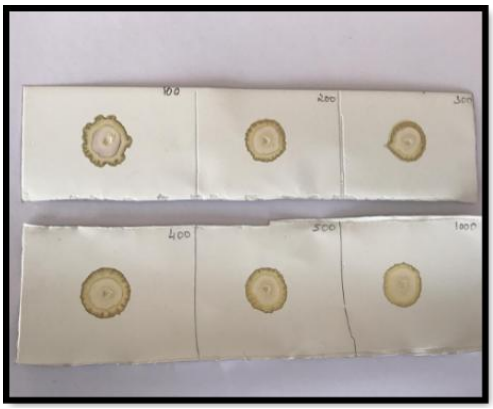

Figure 3: DOT PLOT assay

CONCLUSION

The multiple activities of the plant based medicinal preparations meant for varieties of inflammatory diseases offer enormous scope for combating the threat of diseases. The current examination was intended to create more secure, compelling and practical mitigating natural mix to control oxidative pressure related sicknesses as new options in contrast to synthetic medications. The synergistic cardio protective potential of herbal combination of two medicinal plants was assessed through curative and preventive mode of treatment. Present study shows that poly-phenols content in the 50% hydroethanolic polyherbal crude is high and these extracts exhibit strong antioxidant activities compared to that of the standard compounds. The outcomes would assist with finding out the power of the unrefined concentrate as potential wellspring of normal cell reinforcements. It can be used for minimizing or preventing lipid oxidation in pharmaceutical products and retarding the formation of toxic oxidation products and also been used for the treatment of a variety of diseases. It is an effectively accessible plant for common cures. Further studies on definitive mechanisms of its chemotherapeutic activities and potential effects in vivo are warranted.

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