Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(4):315-325

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

In-Vitro Antioxidant and Cytotoxicity Analysis of Traditional Formulation

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ABSTRACT

The main focus of this study is to prove the therapeutic potential of Traditional formulation (TF) by investigating antioxidant activity in different in vitro models and cytotoxic effect of formulation in normal cell line. In vitro free radical scavenging activity of extracts were studied for DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS (ABTS) 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid, Superoxide, hydroxyl, lipid peroxidation. Total antioxidant assay, reducing power assay and FRAP (Ferric reducing/antioxidant power) assay were also studied. Cytotoxicity study of the formulation was evaluated on HEK 293 cell line and cell viability was determined using MTT assay. The Traditional formulation showed significant and concentration-dependent antioxidant and free radical scavenging activities. Consequently, the formulation exerted lipid peroxidation inhibitory effect by 79.72% as compared to standard ascorbic acid (89.68%). Moreover,formulation has significant scavenging activities of DPPH, nitric oxide, superoxide, ABTS and hydroxyl free radicals in a concentration - dependent manner by 80%, 81.4%, 72.4%, 78.4% and 80.4%, when compared to the standard compound ascorbic acid by 78.4%,77.5%,70.4%,74.3% and 81.3% respectively. Cytotoxity studies revealed non toxic nature of a traditional formulation on experimental cell line. Our studies suggest that TF contain medicinally important secondary metabolites which have significant antioxidant efficacy and non-toxic in nature. However, the in vivo safety needs to be thoroughly investigated in experimental rodent models prior to its practical application.

Keywords: Traditional formulation; Antioxidant; Cytotoxicity

INTRODUCTION

Traditional system of medicine plays a vital role in the health care of ancient and modern humanities against conventional system of medicine [1]. It contains a rich source of biologically active antioxidant compounds like flavonoids, phenolic compound, anthocyanin. Antioxidants have known to protect the biological system through inhibition or prevention of oxidation stress produced by reactive oxygen species in metabolic activities or by environmental factors [2,3]. Free radicals released due to cellular damage leads to many diseases such as liver cirrhosis, atherosclerosis, cancer, aging, arthritis, diabetes, etc., and the antioxidant ability compounds scavenge free radicals create a great prospective in improving these disease processes. Our body has in built mechanism to decrease the free radical induced damage by endogenous enzymes such as superoxide dismutase, glutathione peroxidase, catalase and other vitamins like α-tocopherol, ascorbic acid, biotin etc. Therefore the expedition for exogenous antioxidants is persistent. Most of the authors correlated the antioxidant potential of the plant with their phenolic constituents [4]. Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals. They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers [5].
The liver is one of the vital metabolic organs of the body, responsible for hundreds of chemical actions for the body needs to survive. It plays an important role in human body as it maintains several biochemical pathways

such as body defense against diseases, energy production, and source of nutrition [6]. Oxidative-stress plays a foremost role in the progress of liver diseases. The liver injury is made by the various noxious agents produced by chemicals, viruses or by their bio-activation to chemically reactive metabolites.In modern system of medicine, there is lack of a trustworthy synthetic liver defensive drug. Hence, natural source of medicine are considered to be effective for the treatment of liver disorders [7]. Hence the present work aims to evaluate the antioxidant activity and cytotoxicity effect of traditional formulations (TF), which were used traditionally for treating liver disorders.

MATERIALS AND METHODS

Preparation of Traditional Formulation (TF)

Traditional formulation(TF) consists of 7 ingredients, viz., *Curcuma longa Terminalia chebula, Terminalia belerica, Emblica officinalis, Sphagneticola calendulacea Phyllanthus amarus and Cuminum cyminum.*The formulation was prepared as per the Traditional *Siddha* System of medicine mentioned in the text [8]. All the ingredients were powdered separately, passed through 100 # sieve and then mixed together in equal proportions to get uniformly blended formulations. 10 g of TF was boiled in 500 ml of distilled water for 1 hour. The filtrate was evaporated to dryness under vacuum at 50°**C**-55°**C** using a rotatory evaporator under reduced pressure. Evaporated extracts were reconstituted in water [9].

Chemicals and Reagents

The Gallic acid and Folin-ciocalteau reagent were purchased from Sigma Chemical Co, St. Louis, MO, USA.The standard L-ascorbic acid was obtained from Hi-Media lab.1,1-iphenyl-2-picryl hydrazyl (DPPH), Nitro blue tetrazolium (NBT), phenazine metho sulfate, 2,2'-zombies (2-methypropionamidine) dihydro chloride (AAPH), 2,4,6-tripyridyl-strain. All other chemicals and reagents, all of analytical grade, were from E. Merck (Darmstadt,Germany), unless stated otherwise.

In vitro Antioxidant Assays

The *in vitro* antioxidant assays were carried out by preparing the formulation (1 mg/ml) in 95% methanol and then making its serial dilutions. The specific protocol was followed for finding specific scavenging activities of the formulations.

Total Antioxidant Capacity (Phosphomolybdenum Assay)

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the modified form of Prieto et al. [10]. 0.5 ml of aqueous extract of the traditional formulation (TF) and standard Ascorbic acid was mixed with 2 ml of reagent solution (0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mm ammonium molybdate). The tubes were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using spectrophotometer against blank after cooling.The antioxidant activity is expressed as the number of equivalents of ascorbic acid. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Reducing Power

The reducing power of the extract was determined by the revised method of Oyaizu [11]. 1.0 ml of different concentration of formulation (0.2 to 1.0 mg/ml) was mixed with 2.0 ml of a 0.2 M phosphate buffer (pH 6.6) and 2.0 ml of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 min. Afterwards, 2.0 ml of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 3000 rpm for 10 min.To this 2.0 ml of aliquot of the upper layer,added 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride and absorbance was measured at 700 nm. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Ferric Reducing Antioxidant Power (FRAP) Assay

The determination of the total antioxidant activity (FRAP assay) in the Traditional formulation extract is a modified method of Benzie and Strain [12]. The stock solutions was prepared from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM Hydrocholoric acid and 20 mM $FeCl_3 \bullet 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml of acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \bullet 6H_2O$. The temperature of the solution was raised to 37°C before use. 'TF'extract (200 µl) was allowed to react with 2800 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 NM. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM Fe (II) /g dry mass and compared with ascorbic acid. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) Radical Scavenging Assay

DPPH scavenging activity of Traditional formulations was determined by the revised method of M.Blois [13]. A volume of 100 ml of methanol was used as solvent for 24 mg of DPPH and the stock was kept at 20°C temperatures for further utilization. Different concentrations (0.2 to 1.0 mg/ml) of 100 ml of formulation samples were mixed with diluted 3 ml of DPPH. The tubes were thoroughly mixed and placed for 15 min in incubator at room temperature. Ascorbic acid was utilized as a standard to compare the antioxidant activity. Absorbance of the reaction mixture was measured at wavelength of 517 nm. Antioxidant potential was determined by using a formula.

DPPH scavenging activity (%) = = [(ABS control – ABS sample) / ABS control] ×100

Superoxide Anion Radical Scavenging Activity

Superoxide anion radicals scavenging activity of the traditional formulation (TF) extract was determined by the method of Nishimiki et al. [14]. To 1 ml of nitroblue tetrazolium (NBT) solution (100 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (300 µM in 100 mM phosphate buffer, pH 7.4) and 1 ml of extract in methanol (20 to 100 µg/ml) was added. The reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank sample. The decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

% Scavenging activity = [(ABS control – ABS sample) / ABS control] ×100

(ABTS) 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic Acid Radical Scavenging Assay

ABTS radical scavenging activity of aqueous extract of traditional formulation was determined according to Siddhu et al. [15]. ABTS radical was freshly prepared by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM ABTS solution and kept for 15 hrs in the dark. This solution was diluted with distilled water and read absorbance at 734 nm and the same was used for the antioxidant assay. The final reaction mixture of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of ascorbic acid. Similarly, in the test Group 1 ml reaction mixture comprised of 950 µl of ABTS solution and 50 µl of the extract solutions. The reaction mixture was vortexed for 10 seconds and after 5 minutes absorbance was recorded at 734 nm against distilled water using ultraviolet-visible spectrophotometer (Shimadzu UV-1800) and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

% Scavenging activity = [(ABS control – ABS sample) / ABS control] ×100

Nitric Oxide Radical Scavenging Assay

The nitric oxide radical scavenging activity of the traditional formulation (TF) was determined by the method of Garrat [16]. A volume of 2 ml of 10 mM sodium nitroprusside prepared in phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of "TF" at various concentrations ranging from 10 to 50 µg/ml and ascorbic acid at various concentrations ranging from 25 to 200 µg/ml. The mixture was incubated at 25°C. After 2 hours, 0.5 ml of the incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent. The mixture was incubated at room temperature for 30 min, followed by the measurement of absorbance at 540 nm using spectrophotometer (Shimadzu UV-1800).

% Scavenging activity = [(ABS control – ABS sample) / ABS control] ×100

Hydroxyl Radical

The aqueous extract of the traditional formulation against hydroxyl radical was determined as a measure of inhibition of deoxyribose degradation by method of Halliwell et al. [3] with minor modifications [17]. Each reaction mixture contained, the following final concentrations of reagents in a final volume of 1.0 ml: 2-deoxyribose (2.5 µM), Potassium phosphate buffer (pH 7.4, 20 mM), $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM) and L-ascorbic acid (100 µM). The mixtures were incubated for 1 h at 37°C, followed by the addition of 1.0 ml of 1% (w/v) TBA in 0.05 M NaOH and 1.0 ml of 2.8% (w/v) TCA. The resulting mixture was heated for 15 min at 100°C. After cooling on ice, absorbance was measured at 532 nm. Inhibition of 2-deoxyribose degradation expressed in percentage was calculated as per the equation:

% Inhibition activity = = [(ABS control – ABS sample) / ABS control] ×100

The inhibition % was expressed as IC50, which is defined as the concentration (mg/ml) of the herbal formulation required to scavenge 50% of the hydroxyl radical.

Lipid Peroxidation Assay

A modified thiobarbituric acid-reactive species (TBARS) assay [17] was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media [18]. Egg homogenate (500 µl of 10%, v/v in PBS (pH

7.4) and 100 µl of samples were added to a test tube and made up to 1.0 ml with distilled water. Then, 50 µl of FeSO4 (0.075 M) and 20 µl of L-ascorbic acid (0.1 M) was added and all were mixed and incubated for 1 h at 37°C to induce lipid peroxidation. Thereafter, 0.2 ml of EDTA (0.1 M) and 1.5 ml of TBA reagent (3 g TBA, 120 g TCA and 10.4 ml 70% HClO4 in 800 ml of distilled water) were added in each sample and heated for 15 min at 100°C. After cooling, samples were centrifuged for 10 min at 3,000 rpm and absorbance of supernatant was measured at 532 nm. Lipid peroxidation inhibition was calculated as per the equation:

% Inhibition activity = [(ABS control – ABS sample) / ABS control] ×100

Cytotoxicity Assa

Cell line:

The human embryonic kidney cell line (HEK 293) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity.

Cell treatment procedure:

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions [19]. The viable cells were counted using hemocytometer by tryphan blue exclusion method and diluted with medium containing 5% FBS to give final density of 1×10^5 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 h, the cells were treated with serial concentrations of the samples. They were initially dissolved in dimethylsulfoxide and an aliquot of the formulation solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples was served as control and triplicate was maintained for all concentrations.

MTT Assay

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using a micro plate reader. The percentage cell growth was then calculated with respect to control as follows

% Cell Growth = [A] Test / [A] control × 100

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) for three parallel measurements using Graph Pad Prism version 7.0 for windows, Graph Pad Software, San Diego, California, USA.

Statistical analysis was done by Pearson's correlation coefficient analysis and $p < 0.05$ considered significant. The 50% inhibitory concentration (IC50) was calculated from the non linear regression analysis (Graph Pad PrismVersion 7.0) by plotting percentage inhibition versus concentrations.

Percentage (%) inhibition = [(ABS control – ABS sample) / ABS control] ×100

RESULTS AND DISCUSSION

Total Antioxidant Capacity

The total antioxidant activity of the "Traditional formulation" extract was evaluated by the phosphomolybdenum method and compared with the standard solution of ascorbic acid equivalents. The standard curve of ascorbic acid was done by using ascorbic acid concentration ranging from 20 to 100 µg/ml. The following equation expressed the absorbance of ascorbic acid standard solution as a function of concentration represented as $Y = 0.3342X + 0.0793$, $R^2 = 0.9817$ Where Y is the absorbance and X is the ascorbic acid equivalent (mg/g) of aqueous extract of "Traditional formulation" showed an increase in antioxidant capacity with an increase in concentration. Total antioxidant capacity of "Traditional formulation" extract was found to be 402.9 ± 1.94 µg ascorbic acid equivalent (mg/g) of 100 µl extract as shown in Figure 1. The phosphomolybdenum method is an important antioxidant assay based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphomolybdate (V) complex with a maximal absorption at 695 nm and antioxidant capacity of formulation due to its polyphenol content [20].

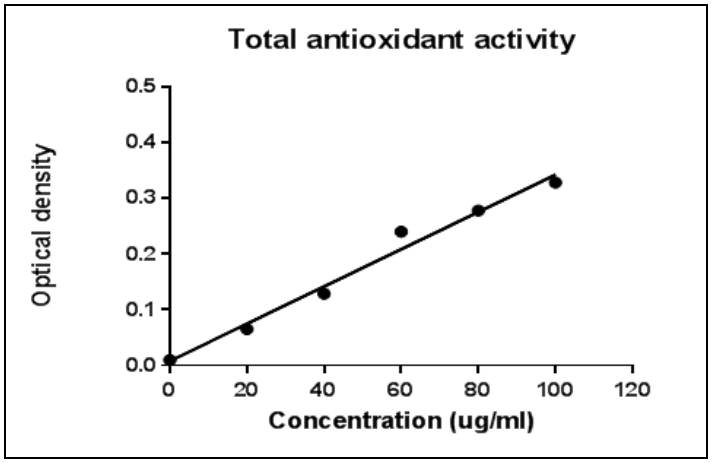


Figure 1: Total antioxidant activity of traditional formulation

Reducing Power Assay

Reducing power of formulation specifies that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes by which they can act as primary and secondary antioxidants [21]. The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe^{3+}/ferricynaide complex to the ferrous form. The product was visualized by forming the intense Prussian blue color complex and then measured at 700 nm. Figure 2 shows the reductive capabilities of the Traditional formulation extract compared to ascorbic acid, a higher absorbance value indicates a stronger reducing power of the sample. Reducing power of formulation extract ranging from 15.395± 0.32 to 41.125 ± 0.69 µg ascorbic acid equivalent/g of the extract.

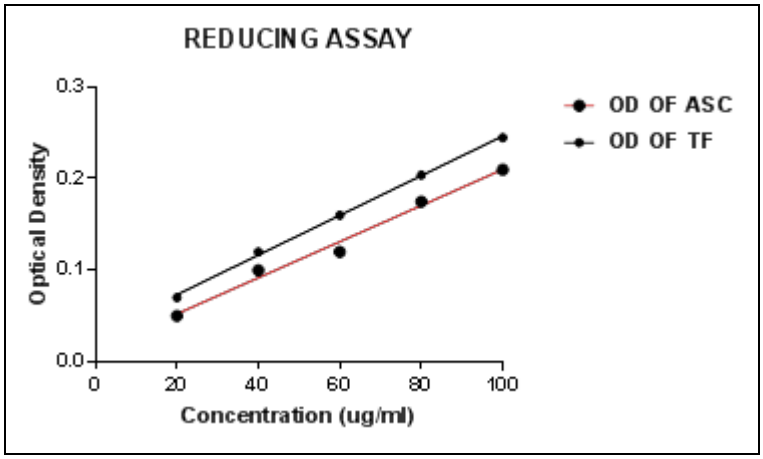


Figure 2: Reducing power activity of traditional formulation and ascorbic acid standard

TF- Traditional Formulation ASC – Standard Ascorbic Acid

Ferric reducing antioxidant power (FRAP) assay:

The reducing potential of an antioxidant was measured by FRAP assay when it reacts with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe2+-TPTZ]. In the present study, the trend for ferric ion reducing activities of "TF"and ascorbic acid are shown in Figure 3. Generally, the reducing properties of constituents exert their activity by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the extract as a reductant in a redox-linked colorimetric reaction [22]. The reducing ability of the extracts was in the range of 241.1 to 466.6 µM Fe (II) /g. The increase in absorbance of formulation is due to the formation of the Fe2+-TPTZ complex with increasing concentration. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols [23]. The aqueous extract of formulation showed increased ferric reducing power with the increased concentration as standard antioxidants. Hence, they should be able to donate electrons to free radicals stable in the actual biological and food system.

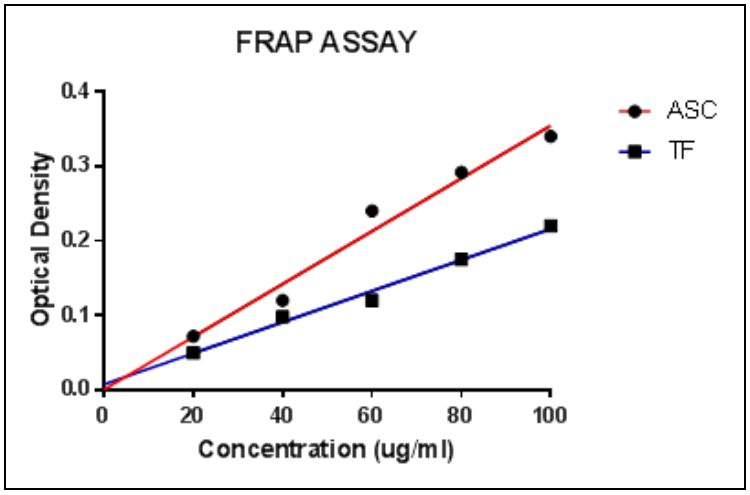
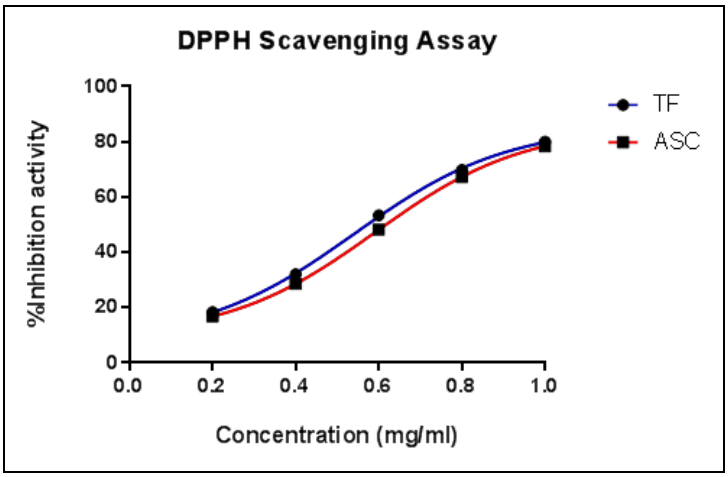


Figure 3: FRAP assay of traditional formulation and ascorbic acid standard

DPPH Radical Scavenging Activity

In the DPPH radical scavenging assay, antioxidants react with DPPH (deep violet color) and convert it to yellow colored α, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical - scavenging potential of the antioxidant. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity [24]. The extract was capable of neutralizing the DPPH free radicals via hydrogen donating activity by 18.4, 32.3, 53.4, 69.8 and 80.1% at concentrations of 0.2 to 1.0 mg/ml, respectively. The IC50 was found to be 5.372 ± 0.07 µg/ml for "Traditional formulation" and for standard ascorbic acid it was found to be 4.721 ± 0.03 µg/ml. There was a significant ($^{*}p<0.05$) relationship between the concentration and percentage inhibition (Pearson's correlation analysis). As shown in Figure 4, DPPH scavenging was increased in a concentration dependent manner compared to ascorbic acid and used as the positive antioxidant control in this investigation.



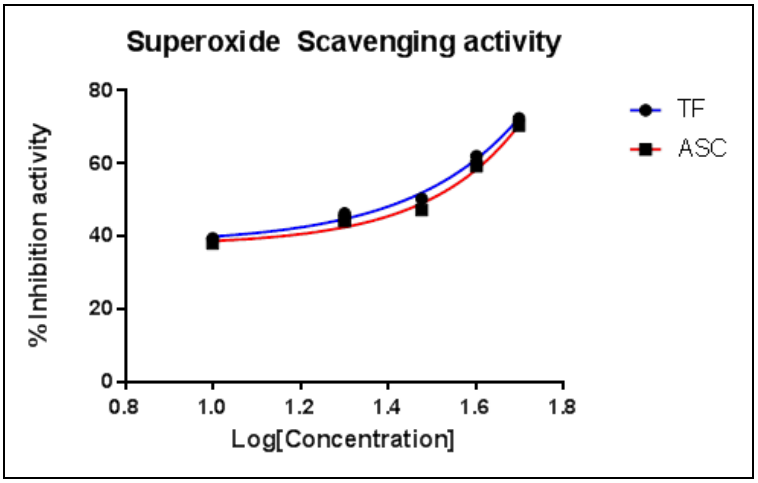
Values are expressed as Mean±Standard deviation(n=3)

Figure 4: DPPH radical scavenging activity of traditional formulation and ascorbic acid standard

Superoxide Scavenging Activity

Superoxide anions are the most common free radicals whose concentration increases under conditions of oxidative stress and are generated either by autooxidation processes or by enzymes and produces free radicalswhic cause cell damage. The superoxide radical is ubiquitous in aerobic cells [25]. Superoxide anion reduces NBT into formazan at pH 7.8 at room temperature and formazan generation is measured by spectrophotometry at 560 nm. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. In this assay, "Traditional formulation" extract exhibited very strong superoxide anion scavenging activity and the result is presented in Figure 5. The superoxide anion radical-scavenging activity of the extract may be due to the presence of phenolic compounds. The IC50 was found to be 38.76 ± 1.97 µg/ml for "Traditional formulation" and for standard ascorbic acid, it was found to be 33.56 ± 1.81 µg/ml. There was a significant ($^{*}p<0.05$) relationship between the concentration and percentage inhibition. The increase in activity is due to increase in number of phenolic hydroxyl groups in the molecule. Superoxide has also been observed to directly initiate lipid peroxidation and plays an important

role in the formation of other reactive species like hydroxyl radicals, which induce oxidative damage in lipids, proteins and DNA. During the process of inflammation, cells of the immune system generate superoxide radicals in which NADPH oxidase plays an important role in induction of vascular complications [26].

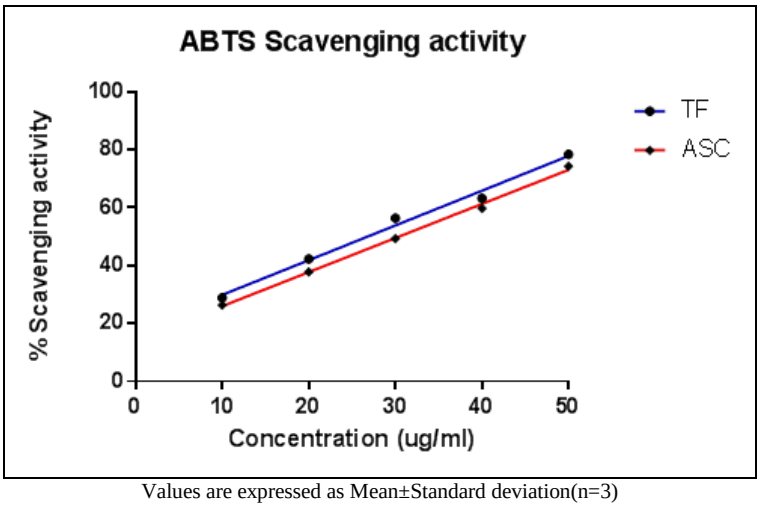


Values are expressed as Mean±Standard deviation(n=3)

Figure 5: Superoxide scavening activity of traditional formulation and ascorbic acid standard

ABTS Radical Activity

Aqueous extract of Formulation exhibited a powerful scavenging activity for ABTS radical cations in a concentration dependent manner. Maximum inhibition was observed with the aqueous extract and standard ascorbic acid ranges from 28.9 to 78.4% at 10-50 µg/ml as shown in Figure 6. The IC50 was found to be 23.28 ± 1.71 µg/ml for "Traditional formulation" and for standard ascorbic acid, it was found to be 27.24 ± 1.42 µg/ml. There was a significant ($^{**}p<0.01$) relationship between the concentration and percentage inhibition. This property may be credited to the presence of polyphenols and flavones in the extract of Traditional formulation. Studies report that the high molecular weight phenol compound have more abilities to quench free radicals (ABTS) and their effectiveness depends on the molecular weight, the number of aromatic rings, and nature of hydroxyl group's substitution than the specific functional groups [27]. Free radical (ABTS) scavenging activity of the traditional formulation (TF) extracts is due to the presence of high molecular weight phenolic compounds.

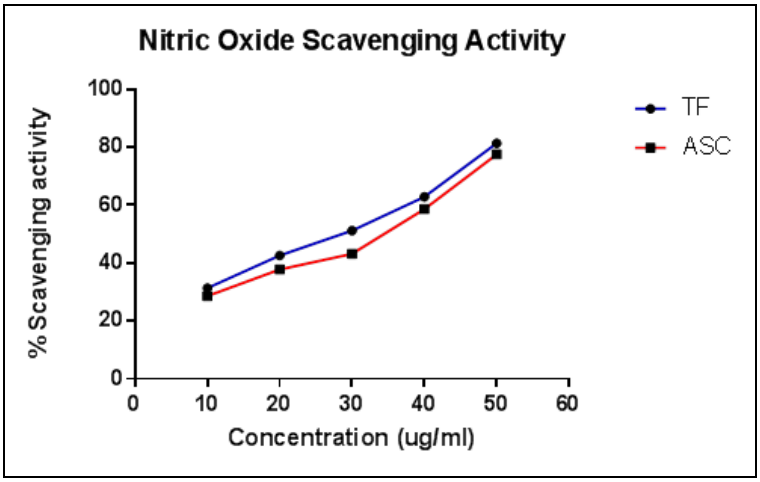


Values are expressed as Mean±Standard deviation(n=3)

Figure 6: ABTS radical activity of traditional formulation and ascorbic acid standard

Nitric Oxide Radical Scavenging Assay

"Traditional formulation" effectively reduced the generation of NO. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 150 min resulted in the generation of NO. The IC50 was found to be 32.59 ± 1.5 µg/ml for "Traditional formulation" and 190.46 ± 3.87 µg/ml of ascorbic acid (Figure 7). There was a significant ($^{**}p<0.01$) relationship between the concentration and percentage inhibition. It has been reported that it reacts with ·O2 radical to form peroxynitrite radicals (ONOO-) that cause toxicity to bio molecules such as proteins, lipids and nucleic acids [28]. ·O2 further decomposes into singlet oxygen and HO· that result in massive mitochondrial damage. Traditional formulation (TF) significantly inhibits generation of NO· and HO· radicals in a dose-dependent manner [29]. These observations further highlights the importance of "Traditional formulation" in preventing physiological deleterious caused by NO· and ·O2 radicals.

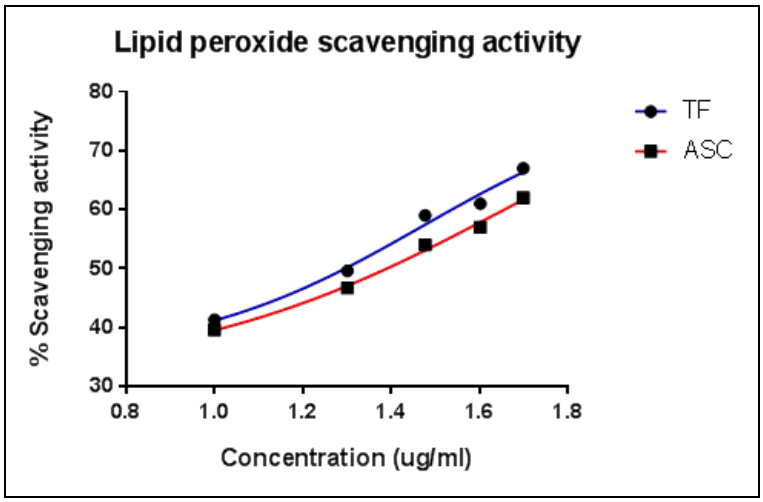


Values are expressed as Mean±Standard deviation(n=3)

Figure 7: Nitric oxide radical scavenging assay of traditional formulation and ascorbic acid standard

Lipid Peroxidation Inhibition Activity

Traditional formulation also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was observed with the aqueous extract and ascorbic acid standard with inhibition percentage 34.71 - 79.72% and 31.3 - 89.68% at 50 µg/ml, with IC50 values of 29.64% for TF and 46.6% for Ascorbic acid as shown in Figure 8. There was a significant ($^{**}p<0.01$) relationship between the concentration and percentage inhibition. This inhibition of lipid peroxidation possibly either due to chelation of Fe. Iron also plays the major role in the formation of lipid peroxidation in the body. The process of lipid peroxidation has been suggested to proceed via a free radical chain reaction [30], which has been associated with cell membrane damage. This membranous damage has been suggested to contribute to various diseases including liver necrosis and diabetes. It is possible that the high level of inhibition of lipid peroxidation displayed by the ethyl acetate fraction is related to the presence of phenolic compounds, which have been correlated with antioxidant activity [31].



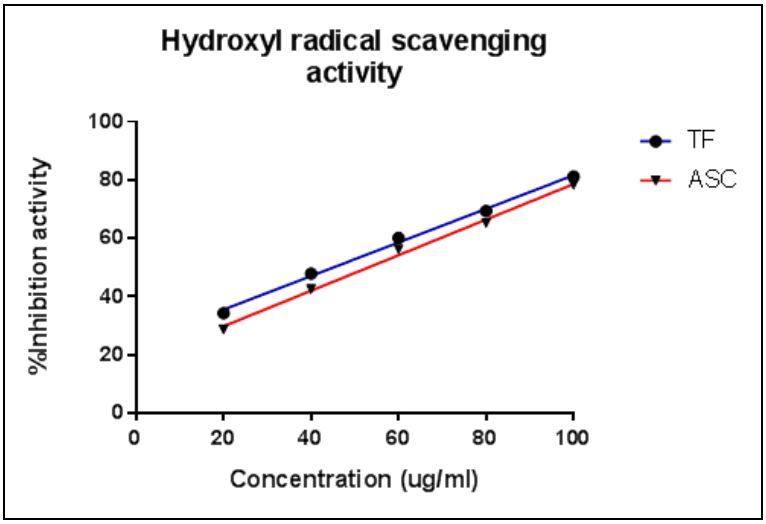
Values are expressed as Mean±Standard deviation(n=3)

Figure 8: **Lipid peroxidation inhibition activity of traditional formulation and ascorbic acid standard**

Hydroxyl Radical Scavenging Activity

The effect of the aqueous extract of Traditional Formulation on hydroxyl radicals generated by Fe^{3+}ions was measured by determining the degree of deoxyribose degradation, an indicator of thiobarbituric acid–malonaldehyde (TBA-MDA) adduct formation. Hydroxyl radicals (˙OH) are the major active oxygen species causing oxidation of polyunsaturated fatty acid in food and enormous cellular and tissue damage [32]. As shown in Figure 9 the extract inhibited hydroxyl radical induced deoxyribose degradation in a concentration dependent manner with a maximal inhibition of 81.4 ± 1.6% observed at a concentration of 50 µg/ml of extract. There was a significant ($p<0.05$) relationship between the concentration and percentage inhibition (Pearson's correlation analysis) with R^2 value of 0.9961 of TF and 0.9954 of standard ascorbic respectively. The antioxidant(s) in the extract could be acting as chelators of the Fe^{3+} ions in the system thereby preventing them from complexing with the deoxyribose, or simply donating hydrogen atoms and accelerating the conversion of H_2O_2 to H_2O [33].

The observed ability of the extracts to scavenge or inhibit. OH radical indicates that the extracts significantly inhibit lipid peroxidation in concentration dependent manner.



Values are expressed as Mean±Standard deviation(n=3)

Figure 9: Hydroxyl radical scavenging activity of traditional formulation and ascorbic acid standard

Cytotoxicity Assay

Cytotoxicity studies with normal cell culture systems of polyherbal or folk medicinal plant extracts has not been studied extensively and has much importance for the safety evaluation of any herbal preparation [33]. Therefore, the objective of this study was to evaluate the potential cytotoxic activity of Traditional formulation against human embryonic kidney cell line (HEK 293). *In vitro* assay of aqueous extract of traditional formulation was carried out for their confirmation of cytotoxic effect on HEK 293 cell line. Percentage of viable cells can be obtained by performing trypan blue dye exclusion technique. The cytotoxicity activity is carried out by using MTT assay. The MTT assay is a rapid and highly accurate colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through an enzymatic reaction on the reduction of MTT to formazan. The toxicity and cell viability of Traditional formulation (TF) had been shown in Figures 10 and 11. Cell lines derived from NCCS, Pune were free from any kind of bacterial and fungal contamination. Percentage cell viability of human embryonic kidney cell line (HEK 293) was carried out by using trypan blue dye exclusion technique gives the result of 96.5%, which is most suitable to perform cytoxicity study.

The cytotoxicity study was carried out for aqueous extract of formulation. These extracts were screened for its cytotoxicity against human embryonic kidney cell line (HEK 293) at different concentrations to determine the IC50 (50% growth inhibition) by MTT assay (IC50 = 12.75 µg). Results of the % viability of human embryonic kidney cell line (HEK 293) of Traditional formulation were graphically represented in Figure 10. The percentage viability was found to be increasing with decreasing concentration of formulation. The above study shows that the Traditional formulations do not have any significant cytotoxicity on the normal human embryonic kidney cell line (HEK 293) as shown in Figure 11. Thus, the toxicity of the plant has to be studied for further exploration of various biological activities. The study was conducted to assess the possibility of this formulation extract to have a potent non-cytotoxic potential.

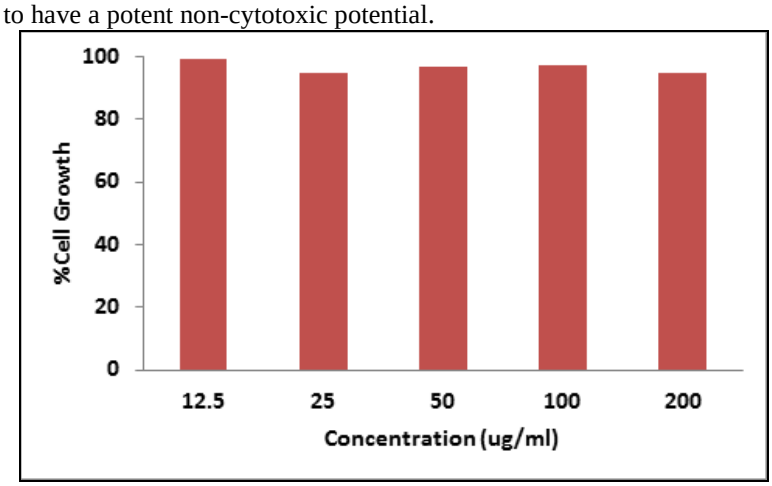


Figure 10: The percentage of cell viability on traditional formulation

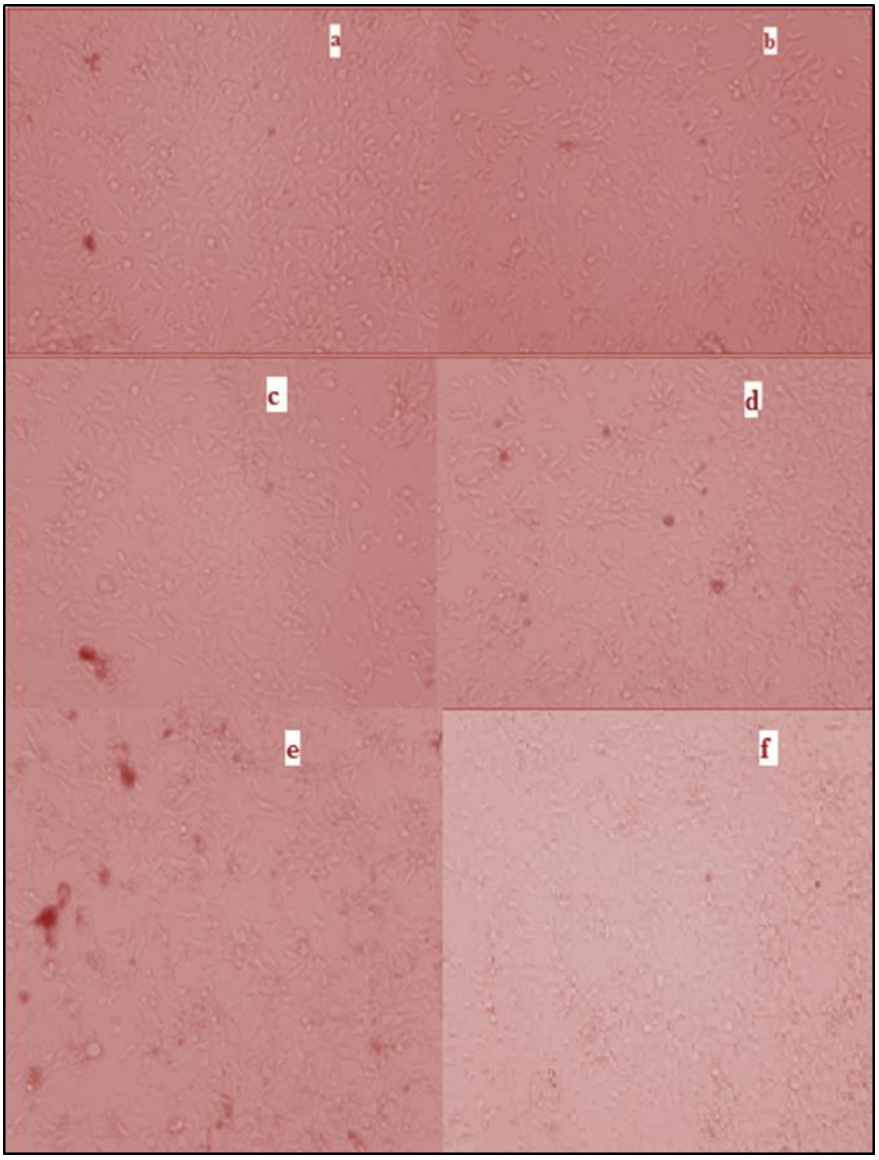


Figure 11: Morphological observation of HEK 293 cell line treated with different concentration of aqueous extract of traditional formulation; a: 12.5 µg of TF; b: 25 µg of TF; c: 50 µg of TF; d: 100 µg of TF; e: 200 µg of TF; f: Control (HEK 293 cell line)

CONCLUSION

The present study concludes that Traditional formulation exhibited effective antioxidant and free radical scavenging activities. Moreover, the hydrogen donating ability of formulation has been proven through the assessment of reducing power ability and radical scavenging activities. It also exerted lipid peroxidation inhibition activity. The cytotoxicity study proves the formulation to be nontoxic to normal cell line. These results prove the efficacy of the drug as a prominent source of natural antioxidant for hepatoprotection. However, the *in vivo* safety needs to be thoroughly investigated in experimental rodent models prior to its practical application.

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