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In vitro antioxidant potential of Shemamruthaa (a herbal formulation) and its anticancer activity in MCF-7 cell line

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Abstract:

Current study investigates the free radicals scavenging activities of methanolic and aqueous extracts of *Shemamruthaa* (*Hibiscus rosa sinensis, Emblica officinalis* and honey in definite ratio) and its anticancer activity in breast cancer (MCF-7) cell line ex vivo. The free radicals scavenging activities of the Shemamruthaa extracts *viz.* 1,1-diphenyl -2- picrylhydrazyl (DPPH), nitric oxide, superoxide and hydroxyl radicals scavenging activities, inhibition of lipid peroxidation, reducing power and total antioxidant activities were measured. Furthermore, the *in vitro* anticancer activities were evaluated for the whole formulation *Shemamruthaa* and its plant constituents. Results of the study indicated that the methanolic extract hold higher radical scavenging activities, reducing power and total antioxidant activity with higher levels of total flavonoids and total phenols than the aqueous extract. *Shemamruthaa* showed superior anticancer activity against MCF-7 cells than the other two plant constituents. The IC_{50} values were found to be 100, 150 and 175 μg/ml for *Shemamruthaa*, *Hibiscus rosa sinensis* and *Emblica officinalis*, respectively. Potent antioxidant from natural sources is of great interest to replace the use of synthetic antioxidants. The results of the study indicate that the Shemamruthaa might be a good candidate for further investigation in developing new antioxidants and anticancer agents.

Key Words:

Shemamruthaa, Antioxidants, Free radicals, MCF-7 cell line, Lactate dehydrogenase, Breast cancer.

Introduction

Reactive oxygen species (superoxide anion radical, hydroxyl radical, and hydrogen peroxide) can cause oxidative damage to macromolecules such as DNA, proteins, lipids and small cellular molecules[1]. They are known to be the underlying cause of oxidative stress which is grossly implicated in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging and metabolic syndrome[2]. Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage[3]. However the innate defense may not be enough for severe or continued oxidative stress. Recently, interest has been increased considerably in finding naturally occurring antioxidants in food [4] or medicinal flora to replace synthetic antioxidants[5]. Meanwhile, the ingestion of several synthetic antioxidants such as Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) has been reported toxic to man [6].

Use of plants as a source of medicine has been inherited and is an important component of the health care system. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world [7]. Approximately 20% of the plants have been subjected to pharmaceutical or biological tests in the world[8]. Plant phenolics, in particular phenolic acids, tannins and flavonoids are known to be potent antioxidants and occur in vegetables, fruits, nuts, seeds, roots, flowers and barks[9]. Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxycinnamic derivatives, catechins *etc*.) of plant origin have been reported as free radicals scavengers and inhibitors of lipid peroxidation.

Shemamruthaa (SM) consists of *Hibiscus rosasinensis* flowers, fruits of *Emblica officinalis* and pure honey in definite ratio. Honey was added to the drug, in order to promote intellect and prevent senility and also for longevity[10]. Both *Hibiscus rosa sinensis* and *Emblica officinalis* were independently proved to exhibit a vital role in traditional and modern medicines, because of their wider pharmacological activities. Previous reports from our laboratory have demonstrated the anticancer efficacy and *in vivo* antioxidant activity of Shemamruthaa against 7,12-dimethylbenz[α]anthracene (DMBA) induced mammary carcinoma rats[11, 12].

Hibiscus rosa sinensis L. (family: Malvaceae), commonly known as China rose is a potent herb used in traditional system of medicine[13] and is being used against cough, fever, dysentery, venereal diseases and cancerous swellings [14]. The constituents present in the extract include flavonoids, cyanidine, quercetin, hibiscetin, glycosides, riboflavin, niacin, carotene, taraxeryl acetate, β-sitosterol, campesterol, stigmasterol, ergosterol, citric, tartaric and oxalic acids, cyclopropenoids and anthocyanin pigments[15,16].

The fruits of *Emblica officinalis* (*Phyllanthusemblica* L.; family: Euphorbiaceae) are widely consumed raw or cooked and are also principal constituents of many Ayurvedic preparations. The fruit extract has many pharmacological activities such as inhibition of clastogenicity and mutagenicity induced by heavy metals and protects against radiations[17]. Studies have confirmed the potential antitumour, anticarcinogenic and chemopreventive action of *P. emblica* extracts on various *in vivo* and *in vitro* models[18].

To the best of our knowledge, there was no scientific report to give credence to ethnomedicinal usage of these plants for the management of various ailments. Therefore, the present investigation was undertaken to study the *in vitro* antioxidant and anticancer potentials of the herbal formulation, *Shemamruthaa*.

Material and Methods

The flowers of *Hibiscus rosa sinensis* and fruits of *Emblica officinalis* were obtained from southern part of India (Kanchepuram District, Tamil Nadu) and the pharmacognostic authentication was done by Department of Plant Sciences, University of Madras, Chennai- 600 025. The flowers and deseeded fruits were air dried under shade, pulverized and mixed with pure honey in definite ratio.

Extraction procedure

Methanol and aqueous extracts were selected because they have been reported to be among the best solvents for the extraction of antioxidant compounds. Fifty grams of the fruit of Shemamruthaa was weighed accurately and soaked in 50 mL of the two solvents separately and kept in a dark place for 3 days in a shaker. Carbon dioxide was released frequently. After 3 days, samples were filtered and the filtrates were kept in a water bath at about 40^0C in order to concentrate them. The concentrated filtrates obtained were used for further studies at different concentrations.

2.2. Preliminary Phytochemical Screening

The SM was used for the phytochemical tests, compounds which include tannins, flavonoids, alkaloids, saponins, carbohydrate, phenols, triterponoids and steroids in accordance with the methods[19, 20] with slight modifications.

Determination of DPPH radical scavenging activity

1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) assay was performed as described by Koleva*et al*.,[21]. About 10 µL of different concentrations (10-100 µg/ml) of test sample solution was added to 190 µL DPPH (150 µM) in ethanol solution. Latter vortexes and they were incubated for 20 minutes at 37°C. The solvent alone acts as a blank. The decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was measured at 517 nm and the percentage inhibition was calculated. Butylated hydroxytoluene (BHT) and vitamin C were used as standards. The IC_{50} values were determined as the concentration of the test mixture that gave 50% reduction in the absorbance from a control blank.

%inhibition = (Absorbance of control-Absorbance of test sample) / Absorbance of Control X 100

Determination Lipid Peroxidation inhibition assay

A 10 % liver homogenate (chicken liver homogenate purchase from local butcher shop) was prepared using ice-cold potassium chloride (0.15 M) in a Teflon tissue homogenizer and the protein content was adjusted to 500 mg/ml. In the control system, 1 ml of tissue homogenate, the lipid peroxidation was initiated by the addition of ferrous sulphate (25 mM), ascorbate (100 mM) and potassium dihydrogen phosphate (10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37^{o}C for 30 min. In the test system, homogenate was incubated with different concentrations of SM (10-100 µg/ml). The extent of inhibition of lipid peroxidation was evaluated by the estimation of thiobarbutric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm [22]. Butylated hydroxytoluene (BHT) and vitamin C were used as positive controls.

Scavenging of nitric oxide radical activity

Aqueous sodium nitroprusside at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite, which can be estimated by use of Greiss reagent. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with 3 ml of different concentrations (10-100 µg/ml) of SM dissolved in methanol and incubated at 25^0C for 150 min. The samples from the above were allowed to react with Greiss reagent .The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm[23]. Butylated hydroxytoluene (BHT) and vitamin C were used as positive controls. The experiments were repeated in triplicates. The percentage scavenging of nitric oxide radical activity was calculated using the formula as mentioned earlier.

Determination of superoxide anion radical scavenging activity

The assay for superoxide anion radical scavenging activity was based on a riboflavin-light-NBT system[24]. The reaction mixture contained 0.5 mL of phosphate buffer (50 mM, pH 7.6), 0.3 mL riboflavin (50 mM), 0.25 mL phenazinemethosulphate (PMS) (20 mM), and 0.1 mL Nitrobuletetrazolium (NBT) (0.5 mM), prior to the addition of 1mL of *Sample* in different concentration (10-100 µg). Reaction was started by illuminating the reaction mixture with different concentrations of SMextracts using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. The absorbance of the control was determined by replacing the sample with methanol. Butylated hydroxytoluene (BHT) and vitamin C were used as positive controls.

Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the SM extracts was assayed by the method of Halliwell and Gutteridge [25]. The reaction mixture contained 500 mL of 2-deoxyribose (2.8 mM) in potassium phosphate buffer (50 mM, pH 7.4), 200 mL of premixed ferric chloride (100 mM) and Ethylene diamine tetra acetic acid (EDTA) (100 mM) solution (1:1; v/v), 100 mL of Hydrogen peroxide (H_2O_2) (200 mM) without or with the extract (100 mL) in different concentration (10-100 μg). The reaction was triggered by adding 100 mL of 300 mMascorbate and incubated for 1 h at 37^0C. A solution of Thiobarbutric acid (TBA) in 1 mL (1%; w/v) of 50 mM Sodium hydroxide (NaOH) and 1 mL of 2.8% (w/v; aqueous solution) Trichloroacetic acid (TCA) was added. The mixtures were incubated for 15 min in a boiling water bath and then cooled. The absorbance was measured at 532 nm. The absorbance of the control was determined by replacing the sample with methanol. Butylated hydroxytoluene (BHT) and vitamin C were used as positive controls.

Determination of total antioxidant activity (TTA)

The total antioxidant activity was evaluated by the method of Prieto *et al*.,[26]. An aliquot of sample solution / vitamin E (equivalent to 500 mg) was combined with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95^0C for 60-90 min. The samples were cooled at room temperature and the absorbance was measured at 695 nm against the blank in spectrophotometer. The total antioxidant activity was expressed as gram equivalents of vitamin E.

Determination of Reducing Power

The reducing power of extracts was determined according to the method of Siddhuraju and becker [27]. The extract was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferric cyanide ($K_3Fe(CN)_6$) (2.5 ml, 1%). The mixture was incubated at 50^{o}C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then 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 ($FeCl_3$) (0.5 ml, 0.1%), the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The Reducing power was expressed as milligram equivalents to vitamin C.

Total phenolic content

Phenolic contents were estimated by the method described by Singleton and Rossi[28] with some modifications. Briefly, 1 ml of sample (3 mg/ml) was mixed with 1 ml of Folin–Ciocalteau's phenol reagent; after 3 min, 1 ml 35% Na2CO3 was added, and then distilled water was added making the reaction system reach 10 ml. The reaction mixture was mixedthoroughly and allowed to stand for 90 min at room temperature in the dark. Absorbance of all the sample solutions against a blank was measured at 725 nm using the spectrophotometer. Total phenolic contents were expressed as milligram of gallic acid (Sigma) equivalents, GAE/g of extract. Calibration curve was constructed with different concentrations of gallic acid as the standard.

Total flavonoid content

Total flavonoids were measured by the method of Jia *et al*.[29]. One ml of the sample (10 mg/ml) was put into a 10 ml volumetric flask containing 4 ml of distilled water; 0.3 ml of 5%

$NaNO_2$ was added. After 6 min, 0.3 ml of 10% Al $(NO_3)_3$ was added; after 6 min, 2 ml of 1 M NaOH were added. Then, immediately, the volumetric flask was diluted to volume with the addition of 2.4 ml of distilled water and the contents thoroughly mixed. Absorbance of all the sample solutions against a blank was measured at 510 nm, using the spectrophotometer. Total flavonoid contents were expressed on an extract weight basis as milligram of rutin equivalents (RE/g). A calibration curve was constructed with different concentrations of rutin as the standard.

Cell culture

The cancer cell line, MCF-7 was purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cancer cells were maintained in Dulbecco' s modified eagles medium (DMEM) supplemented with 2 mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100 g) were adjusted to 1 mL/L. The cells were maintained at 37^0C with 5% CO_2 in a humidified CO_2 incubator [30, 31].

In vitro cytotoxicity of SM

MCF-7 cells are purchased from National Centre for Cell Science (NCCS), Pune and maintained in DMEM and McCoys 5a medium, supplemented with non-essential amino acids. Cells were cultured at 37^0C in a humidified atmosphere containing 5% CO_2 in a CO_2 incubator [31]. Cells were cultured and ~1 × 104 cells/wells were seeded into 96 well tissue culture plates and incubated for 48 h. MCF-7 100 cells are treated with series of 25–275 µg/mL concentrations of SM, HRS and EO. The treated cells were incubated for 48 h for cytotoxicity analysis. The

cells were then subjected for MTT assay. The stock concentration (5 mg/mL) of MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a yellow tetrazole) was prepared and 100 µL of MTT was added in each SM, HRS and EO treated wells and incubated for 4 h. Purple color formazone crystals were observed and these crystals were dissolved with 100 L of dimethyl sulphoxide (DMSO), and read at 620 nm in a multi well ELISA plate reader (Thermo, Multiskan). OD value was subjected to sort out percentage of viability by using the following formula,

$$\text{Percentage of viability} = \frac{\text{Mean OD value of experimental sample}}{\text{Mean OD value of experimental control (untreated)}} \times 100$$

Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD+. To a set of test tubes, 200 µL of the buffered substrate and 20 µL of sample (supernatant and cell suspensions from each group) were added, and the tubes were incubated at 37^0C for 15 min. After adding 40 µL of nicotinamide adenine dinucleotide solution, the incubation was continued for another 15 min. The reaction was arrested by adding 200 µL of dinitrophenyl hydrazine reagent, and the tubes were incubated for an additional period of 15 min at 37^0C. A total of 20 µL of the sample was added to control tubes in each group after arresting the reaction with dinitrophenyl hydrazine. A total of 1.4 mL of 0.4 N sodium hydroxide solutions was added, and the color was developed. The formation of NADH was measured at 420 nm in a multi-well ELISA (Thermo Multiskan EX, USA) reader against a suitable blank. The percentage of release of LDH from the SM-treated cells was calculated for MCF-7 cells [32].

Result and Discussion

Different concentrations of the methanolic extract and aqueous extracts of SM, ranging from 10 to 100 µg/mL were tested for their antioxidant activity using different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the models.

Phytochemical screening

Phytochemical screening of the methanol and aqueous extracts revealed the presence of alkaloids, flavonoids, carbohydrates, phenols, steroids and glycosides. The results are depicted in Table 1.

DPPH radical scavenging capacity

Fig. 1 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extracts. Butylated hydroxyltoluene (BHT) and vitamin C were used as positive controls presented the highest activity at all concentrations. The IC_{50} values (the concentration with 50% scavenging activity) of scavenging activities on DPPH radical were found to be 36.2 µg/ml, 70.3 µg/ml, 19.3µg/ml and 30.6 µg/ml for methanolic extract, aqueous extract, BHT and Vit C, respectively (Table 2). The DPPH radical-scavenging activity was found to be in the order of BHT >VitC>methanolic extract> aqueous extract. The DPPH free radical scavenging activity is a widely used model for evaluating the free radical scavenging ability of various compounds [1]. One of the mechanisms that antioxidants bring about their action is by scavenging free radicals. Antioxidants, on interaction with DPPH, either transfer electrons or hydrogen atoms to DPPH, converting it into a stable diamagnetic molecule and thus neutralizing

free radical character [33]. DPPH is a compound that consists of a nitrogen free radical which is easily quenched by proton radical scavenger of hydrogen donating antioxidant and subsequently transformed into a non-radical form (DPPH-H)[34].

Nitric Oxide (NO)

Similarly to reactive oxygen species, NO is also implicated in inflammation, cancer and other pathological conditions [35]. NO is a very unstable species under aerobic conditions [36]. Nitric oxide (NO) is a potent mediator of physiological processes, with functions related to cell signalling and vasodilation, protecting organs from ischemic damage, and also shows antimicrobial and antitumor activities. It is a highly reactive and diffusible free radical. The toxicity and damage caused by nitric oxide and superoxide anion is multiplied as they produce reactive peroxynitrite, which leads to serious toxic reactions with biomolecules, like protein, lipids and nucleic acids. The plant products have the property to counteract the effect of nitric oxide formation and, in turn, may be of considerable interest in relation to preventing the ill effects of excessive NO generation in the human body.

Fig 2 shows the strong NO-scavenging activities of the SM and its plants constituents, with IC_{50} values of below 70µg/ml (Table 2). The inhibition reactions were especially rapid at lower concentrations (<50 µg/ml), slowing down considerably at higher concentrations (100 µg/ml). Compared to aqueous extract (63.65%) the best NO scavenging activity was obtained from methanolic extract (80.22%) at the concentration of 100 µg/ml. The percentage of inhibition increased with increasing concentration.

Superoxide radical scavenging capacity

Superoxide anion radical is an initial radical and plays an important role in the formation of other reactive oxygen-species such as hydroxyl radical, hydrogen peroxide or singlet oxygen in living systems[37]. Fig. 3 shows the superoxide anion radical- scavenging activity of SM, HRS, EO, BHT and Vit C. Significant superoxide anion radical scavenging activities were evident at all the tested concentrations of the SM. The scavenging activity increased with increasing concentration. Evidently, the order of the superoxide anion radical- scavenging activity was BHA >Vit C>methanolic extract>aqueous extract. The IC_{50}, as shown in Table2, was found to be 63.8, 70.1, 17.5 and 38.5 µg/ml for methanolic extract, aqueous extract, BHT and VitC, respectively. The results suggest that the compound display scavenging effect on superoxide anion radical generation that could help prevent or ameliorate oxidative damage. Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated [38]. Although O_2 is the primary free radical in the biological system it by itself is quite un-reactive. However, the system converts it into more reactive species viz., H_2O_2 and OH radicals. Superoxide radicals are generated *in vivo* and generally dismutated very effectively by various forms of superoxide dismutase [39]. However, when the cells are exposed to oxidative stress, levels of O_2 may rise substantially, causing oxidative damage to DNA, oxidation of proteins and lipid peroxidation[40]. Hence, this assay provides a highly physiologically relevant way to assess the antioxidant activity of the plant extracts and to compare with standard compounds[41].

Hydroxyl radical

In this study, the Fenton reagent ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH$) as a source of hydroxyl radical was used to test the scavenging activity of methanolic extract and aqueous extract towards hydroxyl radical. As shown in Fig. 4, all the samples exhibited potent or moderate activity in an amount dependent manner. The highest scavenging activity was found in methanolic extract, it showed 64.9% scavenging activity at the concentration of 100 µg/ml, while that of aqueous extract was determined to be 53.23% at the same concentration. The IC_{50} values were found to be 60.1, 74.8, 41.0 and 41.7 µg/ml for methanolic extract, aqueous extract, BHT and Vit C, respectively (Table2). Scavenging of hydroxyl radical (OH^-) is an important antioxidant activity because of the very high reactivity of the OH^-, which enables it to react with a wide range of molecules, such as sugars, amino acids, lipids, and nucleotides, found in living cells [42]. Thus, removing OH^- is very important for the protection of living systems. The hydroxyl radical has highly reactive with aromatic compounds and substances that do not act as electron donors [43]. Hence, the pure antioxidant with the lowest number of hydroxyl groups displayed the highest activity towards hydroxyl radicals.

LPO radical scavenging capacity

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation (LPO) by ferrous sulfate takes place either through a ferryleperferryl complex [44] or through an OH radical by the Fenton reaction [45], thereby initiating a cascade of oxidative reactions. The efficacy of LPO scavenging activity of both methanolic and aqueous extracts and controls were shown in Fig. 5. In the present investigation, the methanolic extract registered the highest LPO scavenging activity (70.32%) while the aqueous extract showed a lower level LPO scavenging activity (64.34%). This may be due to the antioxidants in the SM offer resistance to oxidative stress by numerous mechanisms, including

scavenging free radicals and inhibiting lipid peroxidation, thereby preventing disease [46]. The IC_{50} values were found to be 50.1, 79.6, 20.6 and 42.8 µg/ml for methanolic extract, aqueous extract, BHT and Vit C, respectively (Table2).

Total antioxidant Activity

The phospho molybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. Increase in absorbance indicated the increase of the total antioxidant capacity. The antioxidant capacities of methanolic extract and aqueous extracts were measured spectrophotometrically. Potent antioxidant capacity has been found in all samples. The results shown in Fig 6. The methanolic extract exhibited the highest activity (44.6 ± 2.3 mg α-tocopherol/g) than the aqueous extract (37.87± 1.05 mg α-tocopherol/g).

Reducing power property

It is believed that antioxidant activity and reducing power are related[47]. Reductones inhibit lipid peroxidation by donating a hydrogen atom and thereby terminating the free radical chain reaction [48]. Reducing power, which was used to measure the reductive ability of antioxidants, was evaluated by the transformation of Fe(III) to Fe(II) in the presence of the sample extracts[49]. Antioxidants reduce the Fe^{3+} ferricyanide complex to the ferrous form by donating an electron. The color of the test solution then changes from yellow to different shades of green and blue [50]. The ability to reduce Fe(III) may be attributed to the hydrogen donating effect of phenolic compounds [51]. In the present study, 20.5 ± 1.04 mg and 14.9 ± 1.33 mg of the methanolic extract and aqueous extract showed antioxidant activity against the standard Vit C, as is depicted in Fig 6.

Total Phenol

Phenolics or polyphenolics have attracted considerable attention because of their various biological activities, including antioxidant, antimutagenic, antitumour, antiatherogenic and cardioprotective effects. Many foods and their derivatives, such as tea, cereals, wine, legumes, vegetables, fruit and juices contain phenolic compounds [52, 53]. Total phenols protect the cell constituent's defense system or by directly reacting with free radicals[54]. Phenols show antioxidant property mainly due to their redox potentials and act as reducing agents, quenchers and metal chelates [55]. Rice- Evens and miller [56] reported, the antioxidant activity is based on the number of OH group and on the presence of double bond in the molecule. The total phenolic contents (TPC) in the crude extract and its components of EO and HRS were determined in this work, and the values of TPC were expressed in terms of mg gallic acid/g dry extract. Table 3 showed the TPC values of methanolic extract (156.45 ± 6.69mg gallic acid/g) and aqueous extract (116.43 ±3.893 1.43 mg gallic acid/g).

Total Favonoids contents

Flavonoids are known to be contributors to the antioxidant capacity of fruits and vegetables [57]. Flavonoids are low molecular weight polyphenolic compounds which include flavones, flavonones, isoflavones, flavonols. Flavans-3-ols and anthocyanins[58]. The total flavonoid content (TFC) of the crude SM and its plants components were determined and expressed in mg rutin/ g by comparison with standard rutin treated in the same conditions. Total flavonoid content of methanolic extract and aqueous extract are shown in Table 3. Several

reports have conclusively shown close relationship between antioxidant activity and the amount of total phenolics or total flavonoids [59, 60]

Cytotoxicity Assay

Fig. 7 shows the effect of SM, EO and HRS on human mammary adenocarcinoma MCF-7 cells in culture. This is the first study to report the cytotoxicity of SM against breast cancer cell lines (MCF- 7). MCF 7 cells treated with SM, EO and HRS showed a decrease in cell growth. The dose dependent cytotoxicity was observed in SM treated MCF-7 cells. Fifty percentage of cell death, which determines the inhibitory concentration (IC_{50}) value in the MCF-7 were found to be 100µg/mL, 150 µg/mL and 175µg/mL for SM, EO and HRS, respectively. This enhanced anticancer effect of SM compared to its plant constituents might be attributed to the synergistic action of polyphenols such as flavonoids, tannins, alkaloids, glycosides, saponins, steroids, terpenoids, vitamin C, niacin, pyrogallol, hydroxymethylfurfural, trilinolein and other compounds present in the SM formulation. GC-MS analysis of SM revealed the presence of pyrogallol, 5-hydrxoymethylfurfural, levoglucosan, dipalmitin and trilinolein as the major bioactive constituents. HPLC analysis of SM extract indicated the presence of flavonoids such as gallic acid, quercetin, caffeic acid, rutin and ferulic acid.

Release of lactate dehydrogenase from SM treated cells

Fig 8 The LDH release assay measures cell-membrane injury, whereas the other assays used measure only cell survival; therefore, the latter assays are not suitable for assessing the effect of the release of LDH from cytotoxic chemical-induced cells because they involve the subtraction of variable data on control cells from variable data on treated cells. The percentage of

permeability of LDH through the cell membrane of MCF-7 was calculated using cells treated with the SM, EO and HRS.

The LDH release assay is a sensitive test used to ascertain the cytotoxicity of substances, even at low doses [61]. The present assay was conducted at room temperature for 20 min. Here, the release of LDH was detected in all three fractions, with the best IC_{50} in the range of 50–200 µg/mL. Similarly, SM increased the LDH release in a reproducible dose-dependent manner. This combination approach using this LDH assay provided valuable information about the cytotoxic effects of SM. LDH is a cytoplasmic enzyme that is constantly expressed in most mammalian cells, enabling its use in assessing plasma membrane integrity by detecting the amount of LDH in extracellular space [62].When the longer exposure time (24 h) was used, LDH assay was also able to detect the toxic effect caused by the compounds tested. On the other hand, if the data from in vitro cell viability assays are to be used in predictive toxicology just as such, the nature of the test samples should be taken into account when selecting the assay. It has been suggested that for liposomal preparates, LDH assay would be the most reliable method for toxicity testing [63].

Conclusion

From the above results, it can be concluded that the methanolic extract of the SM showed more potent *in vitro* antioxidant activity, with higher percentage inhibition than the aqueous extract of the SM. Phytochemical analysis revealed the presence of phenolic and flavonoid compounds in the extracts. These compounds may be responsible for the free radical scavenging activity. The high antioxidant activity exhibited by extracts (cell free system) and in MCF-7 cell line which provides justification for the therapeutic use of this plant in folkloric medicine due to its phytochemical constituents. The present data suggest that this extract could be a potential

source of natural antioxidant that could be of great importance for the treatment of radical related diseases. Further studies are in progress to isolate the active principles from the extract and to elucidate the exact mechanism of action of the free radical scavenging effect.

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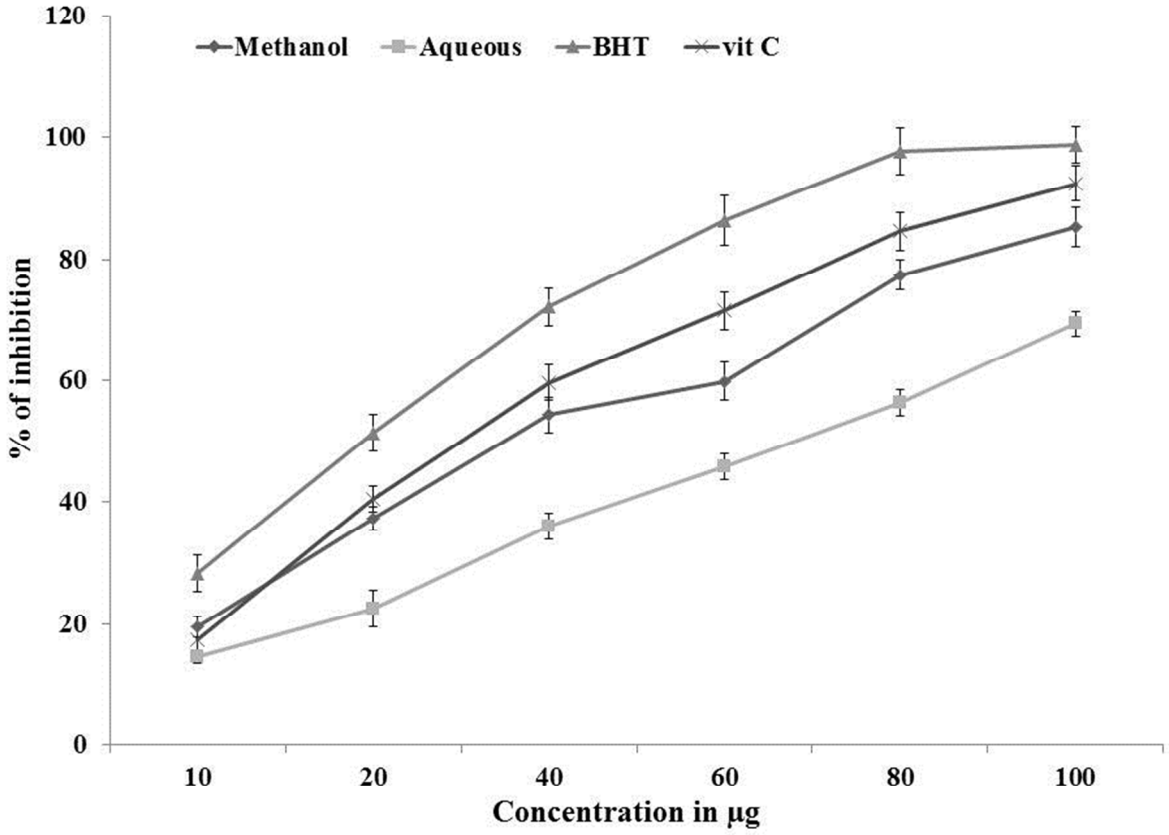

Fig. 1. DPPH radical-scavenging activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations.

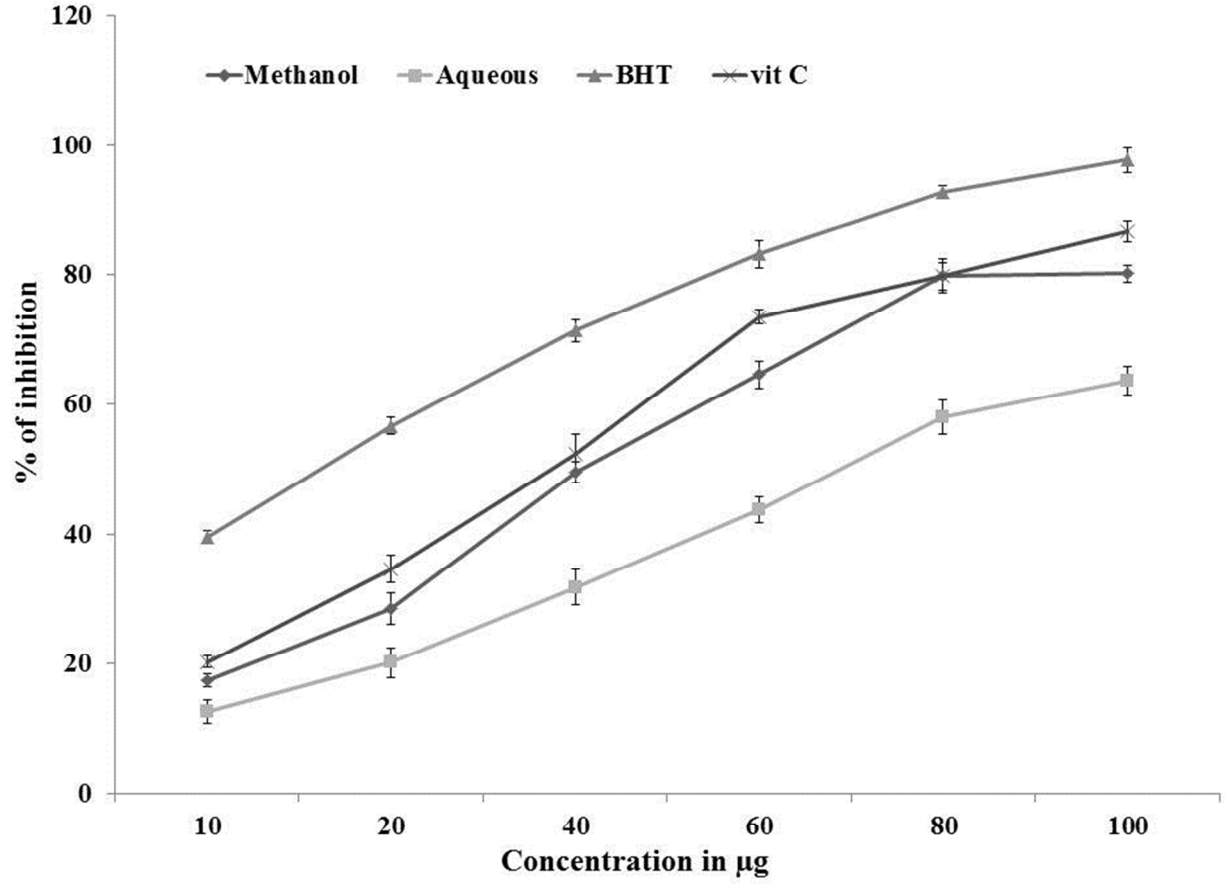

Fig.2. Nitric oxide radical-scavenging activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations.

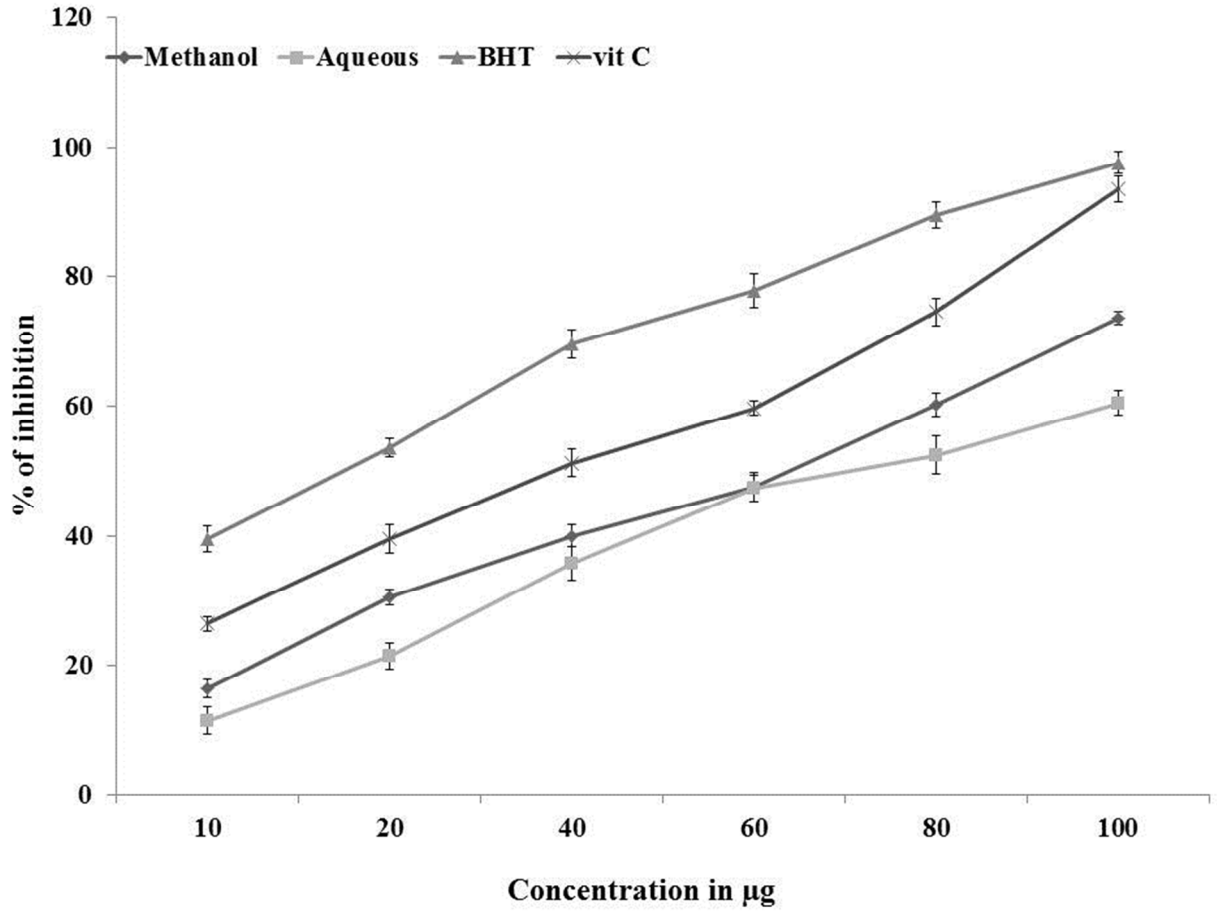

Fig. 3.Superoxide radical-scavenging activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations.

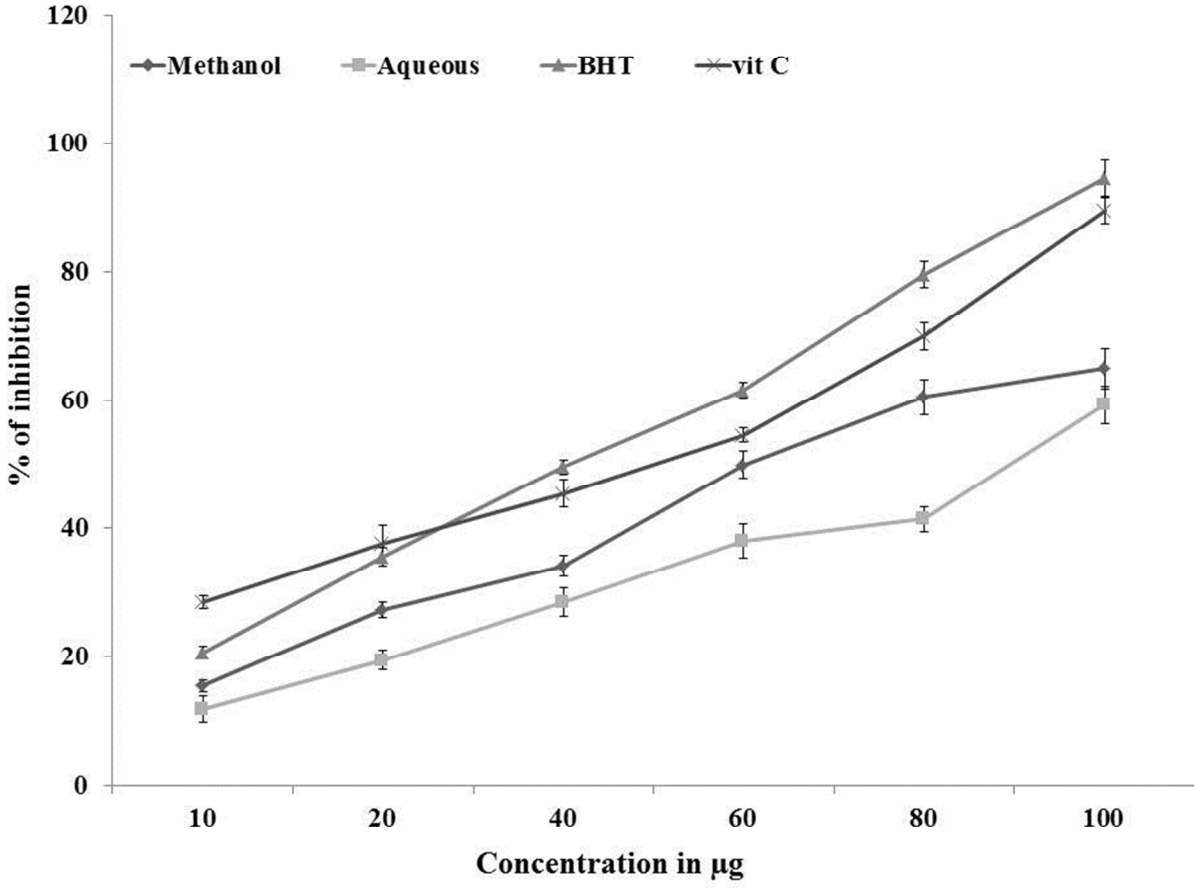

Fig.4. Hydroxyl radical-scavenging activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations.

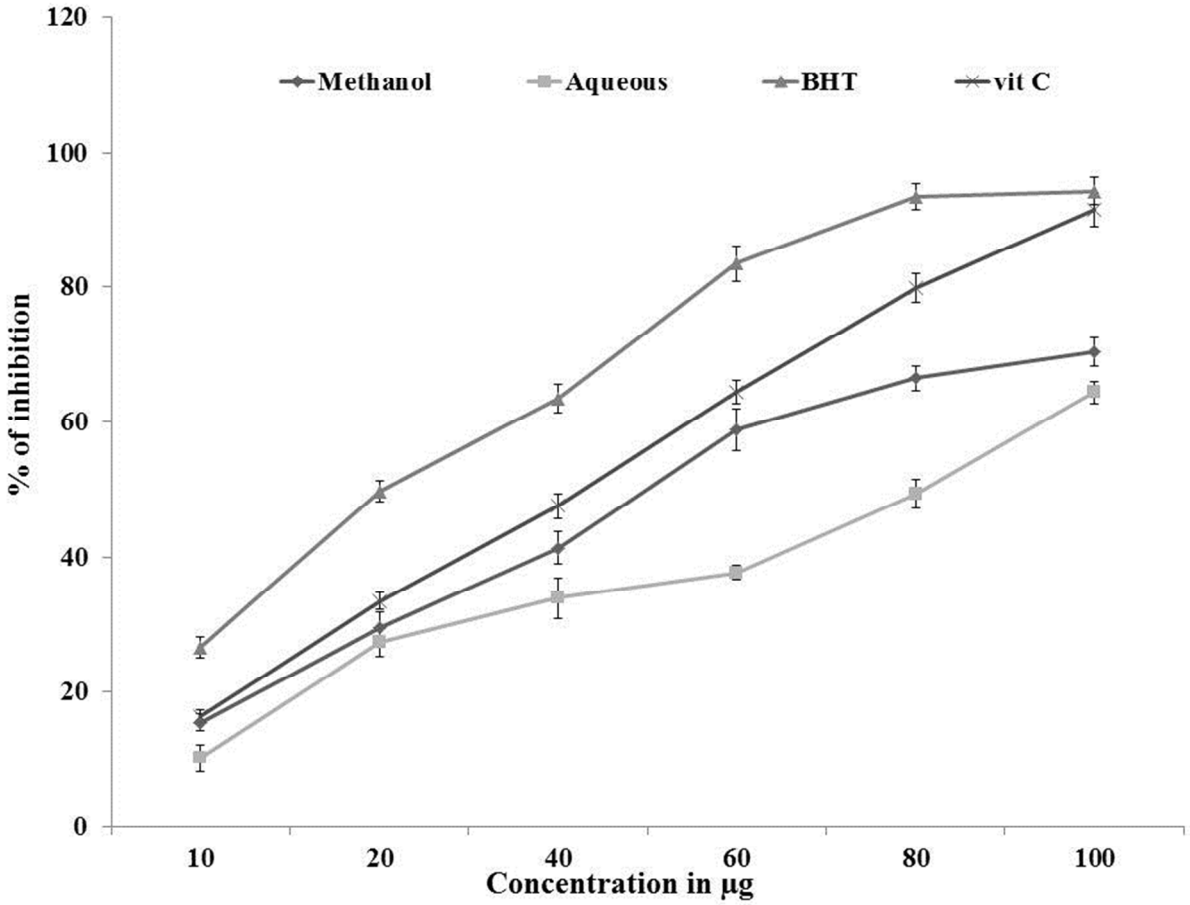

Fig.5. Lipid peroxidation scavenging activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations.

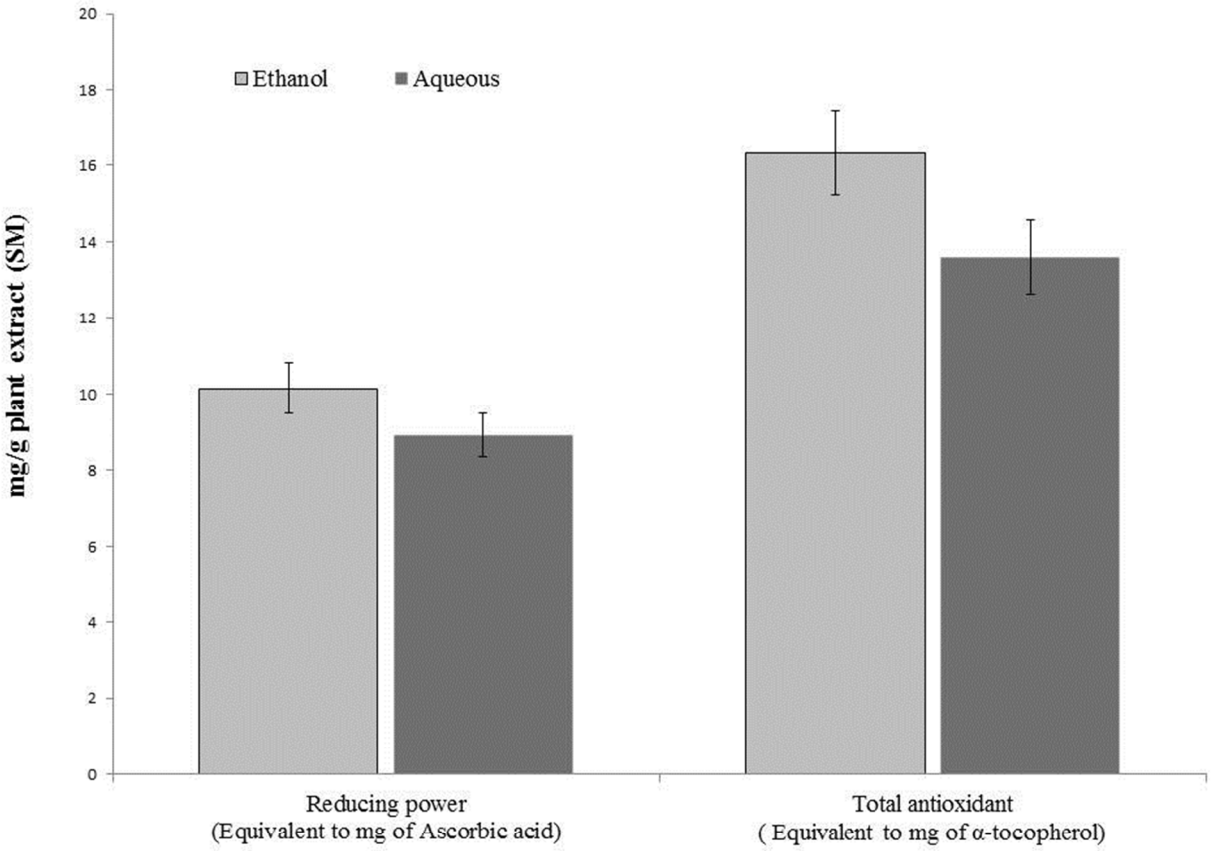

Fig.6. Reducing power and total antioxidant activities of Methanolic extract and Aqueous extracts of SM and Standards (BHT and Vit C). Values are means ± SD of three determinations

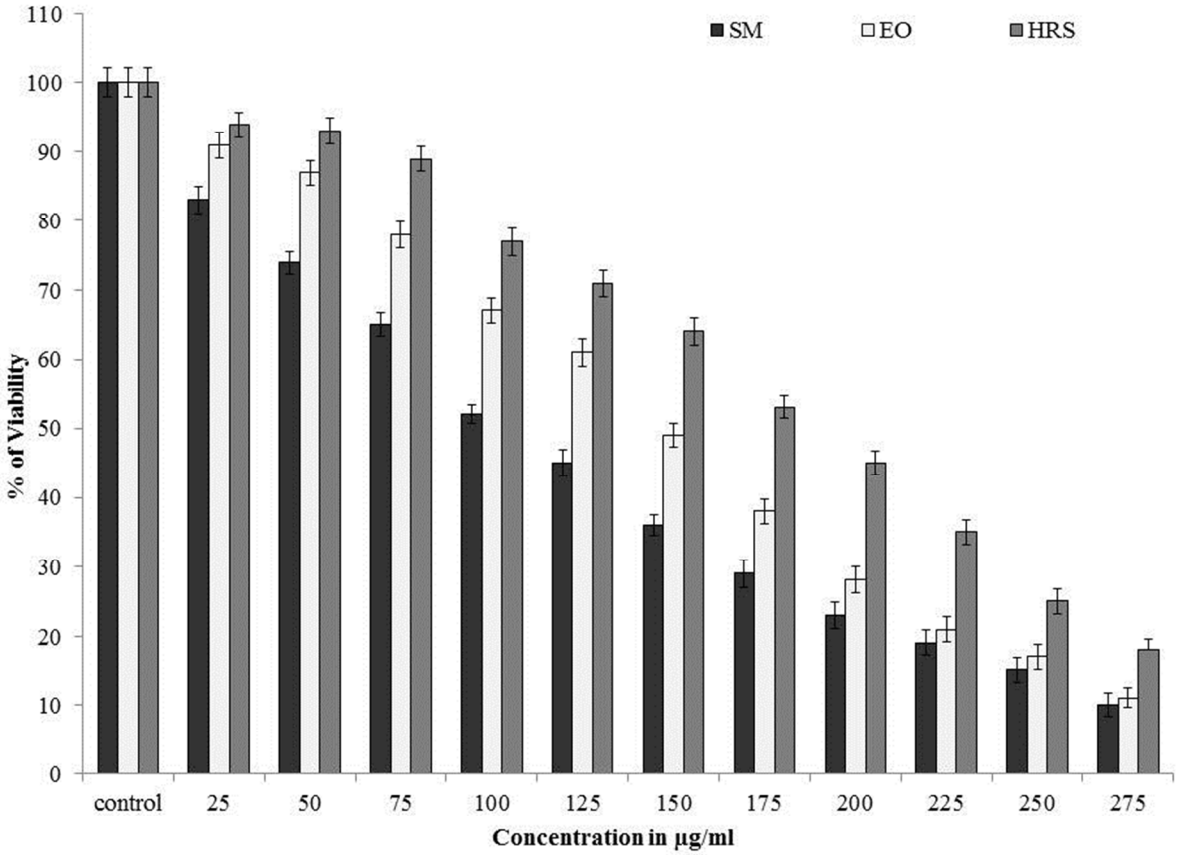

Fig. 7. Effect of SM, EO and HRS cytotoxic activities on MCF-7 cells at 48 h. Values were obtained in triplicate, expressed as the mean ± SD

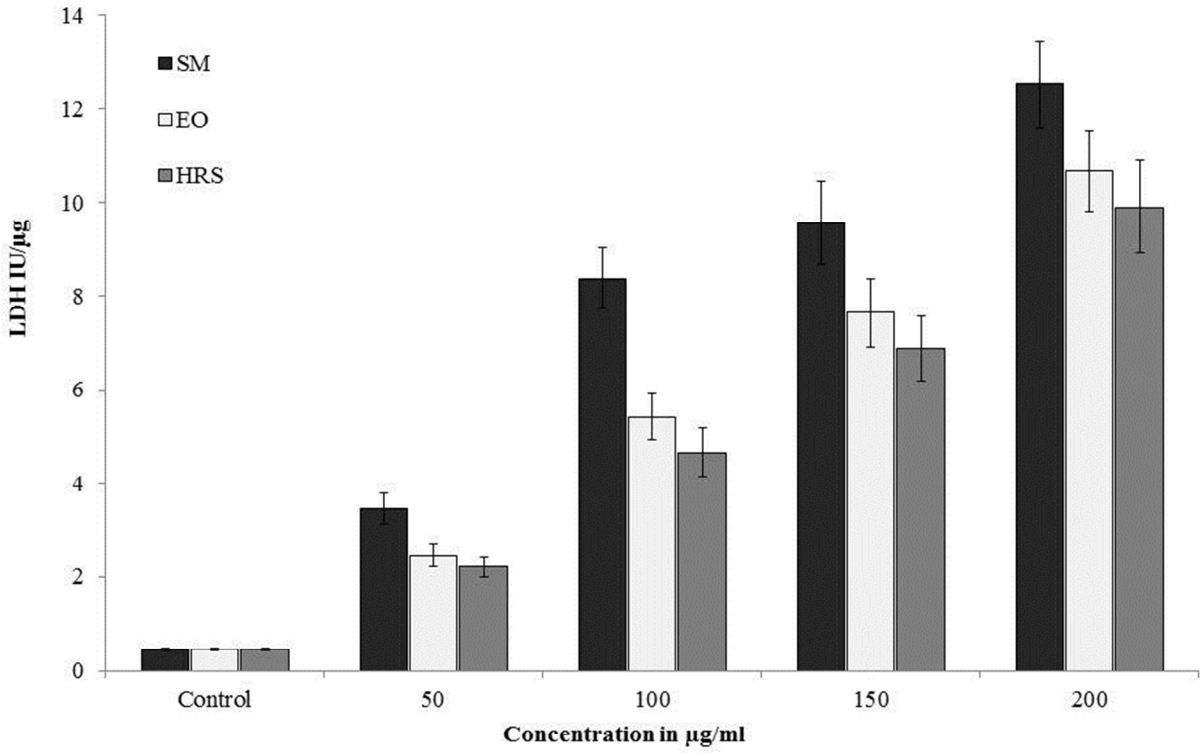

Fig. 8. Release of lactate dehydrogenases after treatment with different concentrations (50–200 µg/mL) of SM, EO and HRS on MCF-7. Values were obtained in triplicate, expressed as the mean ± SD .

Table 1 Preliminary phytochemical screening of Shemamruthaa (SM)

Phytochemical Compounds	Methanolic Extract of SM (MESM)	Aqueous Extract of SM (AESM)
Tannins	++	++
Flavonoids	+++	++
Steroids	++	+
Alkaloids	++	+
Saponins	+	+
Total Phenols	+++	++
carbohydrates	+++	++
Glycosides	++	+

+++ -appreciable amount (positive within 5 min); ++ - Moderate amount) Positive after 5 min within 10 min; + - trace amount (positive after 10 min within 15 min)

Table.2. Radical scavenging activity of MESM, AESM and controls (BHT and Vit C). Values are means ± SD of three determinations.

Compounds	IC 50 (μg/ml)				
	DPPH radical scavenging activity	Nitric oxide radical scavenging activity	Superoxide anion radical scavenging activity	Hydroxyl radical scavenging activity	Lipid peroxidation inhibition activity
Methanol	36.2±0.21	40.3±0.22	63.8±0.43	60.1±0.29	50.1±0.31
Aqueous	70.3±0.64	67.7±0.34	70.1±0.38	74.8±0.43	79.6±0.45
BHT	19.3±0.12	16.6±0.11	17.5±0.15	41.0±0.25	20.6±0.14
Vit C	30.6±0.9	35.9±0.28	38.5±0.28	41.7±0.23	42.8±0.31

Table.3. Total Phenols and total flavonoids of Methanolic and Aqueous extract of SM. Values are means ± SD of three determinations.

Compounds	Total Phenol (Equivalent to mg of Gallic acid)	Total Flavonoids (Equivalent to mg of Rutine)
Methanolic Extract	14.56 ± 0.86	17.37 ± 1.03
Aqueous Extract	11.66 ± 0.67	12.43 ± 0.89