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Antioxidant and cytotoxic properties of three traditional decoctions used for the treatment of cancer in Sri Lanka

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ABSTRACT

Many plant-based treatments are being recommended for the cancer patients by traditional medical practitioners of Sri Lanka. Three such decoctions D1 (*Terminalia bellerica, Terminalia chebula, Phyllanthus emblica* and detoxified *Commiphora mukul),* D2 *(Terminalia bellerica, Terminalia chebula, Phyllanthus emblica,* detoxified *Commiphora mukul, Smilax china and Nigella sativa*) and D3 (*Munronia Pumila, Azadirachta indica, Solanum surattense, Solanum xanthocarpum, Rubia cordifolia, Picrorhiza kurroa, Trichosanthes cucumerina and Pterocarpus santalinus)* were selected to investigate their total polyphenol contents, antioxidant properties and potential anticancer activities. The total phenolic contents of D1 and D2 were ~37 and 30% w/w gallic acid equivalents, where as D3 contains a very low (6%) phenol content. Total free radical scavenging activity (DPPH assay), reducing power and antilipid peroxidation activity (TBARS assay) of each decoction were investigated and these values were compared with ascorbic acid and vitamin E. Decoction D1 and D2 showed higher antioxidant activity and lower EC_{50} values than that of Decoction D3, which strongly associated with their total phenolic content. The MTT assay and LDH assay were used to investigate antiproliferative and cytotoxic activities of these decoctions against the human Rhabdomyosarcoma (RD) cells. The decoctions D1 and D2 showed strong inhibition of cell proliferation against RD cells, where as D3 did not show considerable activity. The chemo preventive and therapeutic potential of the decoctions D1 and D2 can be explained to a certain extent by the results obtained from this study.

KEYWORDS*:* Antioxidant activity, Antiproliferative activity, Cancer, Cytotoxicity, Herbal decoctions, Total phenolic content.

INTRODUCTION

Cancer has become one of the most challenging health problems in the world since it is a major cause of death in both the developed and developing countries (1). Among the possible cause of cancer, damage to DNA and other cellular molecules by reactive oxygen species (ROS) rank, high as a major culprit in the onset and development of disease (2, 3). ROS are an entire class of highly reactive oxygen containing molecules derived from the metabolism of oxygen and in exposure to exogenous sources including nitrogen oxide pollutants, smoking, certain drugs and ionizing radiation. Exposure of ROS to cellular components leads to oxidation of lipids and proteins and alter signal transduction pathways that enhance cancer risk (2, 4).

Of the 121 prescription drugs in use today for cancer treatment, 90 are derived from plant species and almost 74% of these were discovered by investigating a folklore claim(5). The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties(6). Antioxidants have been extensively studied for their ability to prevent cancer in human(7). In searching for novel natural antioxidants, some plants have been extensively studied in the past few years for their antioxidant and radical scavenging components (7-9). In this respect the presence of flavonoids and other polyphenolic compounds have received the greatest attention (10). In Asian countries, like India and Sri Lanka there are well developed traditional alternative medical systems which use various herbal preparations to treat cancer through eliminating the carcinogens from the system, blocking one or more steps in cancer development, retarding the further growth of cancer cells while minimizing side effects of chemotherapy (11). It seems that antioxidants have a dual role in prevention

and cure of cancer. A number of reports show a reduction in adverse effects of chemotherapy when given concurrently with antioxidants (12). However according to the Lamson and Brignell (13), there are only very few known examples, which act as an antioxidant, but has been shown to decrease effectiveness of radiation or chemotherapy *in vivo*.

The traditional cancer treatments used in Sri Lanka is made of decoctions comprising several medicinal plants. The three decoctions investigated in this study D1, D2 and D3 are composed of the following plants. The first decoction (D1) composed of 04 herbal drugs, *Terminalia bellerica, Terminalia chebula, Phyllanthus emblica* and detoxified *Commiphora mukul*. The second decoction (D2) contained *Terminalia bellerica, Terminalia chebula, Phyllanthus emblica,* detoxified *Commiphora mukul, Smilax china* and *Nigella sativa*. The third decoction (D3) composed of 08 plants namely, *Munronia pumila, Azadirachta indica, Solanum surattense, Solanum xanthocarpum, Rubia cordifolia, Picrorhiza kurroa, Trichosanthes cucumerina* and *Pterocarpus santalinus*.

Although the above plant mixtures in the forms of decoction have been prescribed to patients for so many years they have, to date, not subjected to scientific investigation to determine whether these formulation truly have the potential to be of benefit to these patients. However, *Terminalia chebula* (8), *Terminalia bellerica* (14) and *Phyllanthus emblica* (15) have been studied for their anticancer and antioxidant activities as a mixture of three (Thripala) as well as individual components (16,18). Cytotoxic and free radical scavenging activity of *Smilax china*, *Nigella sativa* and *Commiphora mukul* are also reported (19-22). Iddamaldeniya and coworkers (23) has reported the antioxidant and hepatoprotective effects of a decoction containing *Smilax glabra, Nigella sativa* and *Hemidesmus indicus*. Most of the herbs in the third decoction (D3) rarely investigated for their antioxidant and anticancer properties. The objective of the present study is to investigate the antioxidant and antiproliferative properties of the above mentioned decoctions, prepared by the traditional methods in Sri Lanka.

MATERIALS AND METHODS

Plant material

All the dried herbs were obtained from Bandaranayake Memorial Ayurveda Research Institute and from a registered Ayurvedic drug outlet at Colombo (Registered in Ayurveda Drug Cooperation in Sri Lanka). All the herbal materials were identified and confirmed by the Department of Botany, Bandaranayke Memorial Ayurveda Research Institute, Nawinna, Colombo, Sri Lanka. Voucher specimens were deposited at the same institute.

Preparation of Decoctions

The first decoction (**D1**) composed of 4 herbal drugs, *Terminalia bellerica* (fruit), *Terminalia chebula* (fruit), *Phyllanthus emblica* (fruit) and detoxified *Commiphora mukul* (resin)**.** The second decoction (**D2**) contained *Terminalia bellerica* (fruit), *Terminalia chebula* (fruit), *Phyllanthus emblica* (fruit), detoxified *Commiphora mukul* (resin), *Smilax china* (root) *and Nigella sativa* (seeds). The third decoction **(D3)** composed of 08 plants namely, *Munronia Pumila* (leaves), *Azadirachta indica* (bark), *Solanum surattense* (root), *Solanum xanthocarpum* (whole plant) *Rubia cordifolia* (whole plant), *Picrorhiza kurroa* (root), *Trichosanthes cucumerina* (leaves) *and Pterecarpus santalinus* (heart wood). All ingredients were in dried form.

All 3 decoctions were prepared as practiced by the traditional doctors in Sri Lanka. Equal amount of dried herbal components (15 g each for D1, 7.5 g each for D2 and 10 g each for D3) were mixed and boiled with 1400 ml of clean water until the volume get reduced up to 175 ml (1/8th of the original volume). Clay pot with a lid was used to boil the contents. Each decoction was decanted and centrifuged to remove all plant debris. The supernatant was filtered through a Whatmann filter paper (No 01), freeze dried and stored at -20^{o}C until used.

Preparation of stock solutions

Each drug (100 mg) was dissolved in deionized water (10 ml), sonicated (5 mins) and centrifuged (3000 rpm x 5 mins). Freshly prepared solutions were used for each experiment.

Chemicals and equipment

Thiobarbituric acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), 3,4,5-(dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), Eagle's Minimum Essential Medium (EMEM), L-glutamine, Sodium dodecyl sulphate, gallic acid, Folin Ciocalteau reagent, Triton X 100 and trichloroacetic acid were purchased from Sigma chemical Co. (USA). Fetal Bovine Serum and antibiotics (Penicillin /Streptomycin) were purchased from Gibco BRL. LDH enzyme assay kit was purchased from ROCHE diagnostics, Mannheim, Germany. All other reagents and solvents used in the study were of analytical grade. Shimatdzu Biospec 1601 UV visible spectrophotometer (Shimatdzu, Japan) was used to measure the absorbance.

Cell Culture

Human Rhabdomyosarcoma (RD) cells were obtained from Medical Research Institute Colombo 08, Sri Lanka and maintained in Eagle's Minimum Essential Medium supplemented with 10% Fetal Bovine Serum (FBS), Glutamine Penicillin, and Streptomycin in a humidified 5% CO_2 incubator at 37°C.

Determination of total phenolic content

Total phenolic contents were determined using the Folin-Ciocalteu method (24). Briefly, 50 µl of the water extract of each drug was diluted with 450µl of distilled water and 250µl Folin-Ciocalteu reagent (1N). The mixture was allowed to stand at room temperature for 2 minutes and 1.25 ml of sodium carbonate (10%) was added. Absorbance was measured at 760 nm after 45 mins. Gallic acid was used as a standard in the determination of phenolic contents using the calibration curve. The contents of phenolic compounds were expressed as w/w % gallic acid equivalents.

DPPH radical scavenging activity

Free radical scavenging activity of the decoctions was assayed by 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging method described by Blois (25) with slight modifications. Stock solutions (10mg/ml) were diluted with water to obtain required optimum concentrations (0.63, 1.25, 2.50, 3.13, 5.00, 6.25, 12.5 µg/ml concentrations). A volume of 25 µl of the sample was mixed with 475 ml of DPPH (100 µM) in absolute ethanol. Mixture was allowed to stand for 30minutes in dark at room temperature. Deionized water (25 µl) was used as the control. The absorbance (A) was measured at 517 nm compared with the control (Maximum absorbance). Ascorbic acid was used as the standard antioxidant. The scavenging activity of samples was correlated with the intensity of quenching DPPH. The results were expressed as percentage antioxidant index (AI %) using this equation, AI = [(Acontrol -A sample) /A control] x 100. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis of dose response curve plotting between % AI and concentrations.

Determination of anti lipid peroxidation activity

Thiobarbituric acid reactive species (TBARS) assay with slight modifications was used to measure the potential anti lipid peroxidation activity of the decoctions using egg yolk as lipid rich media(26,27). Briefly, 100 µl of egg yolk (10% w/v) in KCl (1.15 %) and 50 µl of sample prepared in water (0.67, 1.34, 2.00, 2.67, 3.34, 4.00 mg/ml concentrations) were added to five snap capped vials. Same amount of de-ionized water was used as the control. Each vial was added with 300µl of 20% acetic acid (pH 3.5) followed by 300µl of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate (SDS). The resulting mixture was vortexed, and then heated to 95°C for 60 min in a heat block. After cooling, to room temperature, 750 µl of butan-1-ol was added to each tube, vortexed, and centrifuged at 3000 rpm for 10 min. The absorbance of the organic layer was measured at 532 nm. The same procedure was repeated with positive control Vitamin E. The absorbance measured was converted to the percentage anti-oxidant index (AI %), using the equation, AI = (1-T/C) x 100 where C is the absorbance value of the fully oxidized control and T is the absorbance of the test sample (27).

Measurement of reducing power

The reducing power of each decoction was determined according to the method used by Dhalwal et al. (28) with slight modifications. Different concentrations (0.05, 0.1, 0.15, 0.2, 0.25 mg/ml) of decoctions (100 ml) were mixed with 250 ml of phosphate buffer (0.2 M, pH 6.6) and 250 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min, and 250 ml of trichloroacetic acid (1%) was added. The resultant mixture was centrifuged at 5,000 *g* for 10 min. The supernatant was mixed with distilled water and FeCl3 (0.1%) at a ratio of 1:1:2 and the absorbance was read at 700 nm. Replicates of six were used for all concentrations of decoction D1, D2 and D3. Increase in absorbance of the reaction mixture is a measure of increase in reducing power.

Cytotoxicity and cell viability assay

Determination of cytotoxicity by lactate dehydrogenase assay

Rhabdomyosarcoma cells were seeded in 12 well plates (NUNC, Denmark) at a density of 2 x 10^5 cells /well and cultured overnight as described by Fotakis and Timbrell, (29). Confluent monolayers were treated with different concentrations of decoction D1, D2, and D3 (50, 100, 150, 200 µg/ml) and incubated in CO_2 incubator at 37°C for 24 hours. LDH activity in the supernatant was measured using the LDH cytotoxcity detection kit (ROCHE diagnostics, Mannheim, Germany) following the manufacturers instructions. The disrupted drug treated cells were again treated with 0.1% Triton X-100 and LDH activity was measured again. The amount of released LDH activity by each decoction was indicated as a proportion of the total LDH activity. Background leakage of LDH was measured after incubation of same density of cells without the drug. Background data were subtracted from the

experimental data appropriately. All experiments were performed in triplicates.

Determination of cell viability by MTT assay

Rhabdomyosarcoma cells were seeded in 12 well plates (NUNC, Denmark) at a density of 2x 10^5 cells /well and cultured overnight. Confluent monolayers were treated with different concentrations of decoction D1, D2 and D3 (50,100,150,200 µg/ml) and incubated in humidified CO_2 incubator at 37°C for 24 hours. Then the media was replaced by new media (2 ml) and 200 µl of MTT (5 mg/ml) was added to each well. Cells were then incubated at 37°C for another 4h, the medium was aspirated carefully and the formazan product was solubilized with 2 ml of 0.05 M HCl in 2-propanol. Absorbance was measured at 570 nm (30).

Statistical analysis

Tests were carried out in replicates in 4-6 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, EC_{50}, was graphically determined by using MS- Windows based software. Results were expressed as graphically or mean ± standard error of the mean (SEM) unless specified.

RESULTS

Extraction yield and total phenol content

The total phenol contents and the extraction yield of the three decoctions (water extract) are shown in Table 1. The extraction yield range from 84 mg/g decoction materials to 250 mg/g decoction materials. The decoctions D1 and D2 have a higher extraction yield when compared to the decoction D3. The total phenolic contents, is also higher in the decoctions D1 and D2 when compared to the decoction D3 as shown in Table 1.

DPPH radical scavenging activity

The free radical scavenging activity of decoctions D1, D2, D3 with increasing concentration are shown in Fig. 1. Ascorbic acid was used as the positive control. The reduction of alcoholic DPPH by D1 and D2 was very high and the scavenging ability increased with increasing concentration. The percentage inhibition at 12.5 µg/ml for D1, D2, D3 and ascorbic acid was 84.8 ± 0.9, 84.0 ± 0.5, 8.5 ±0.1 and 87.7 ± 0.6 respectively. The scavenging effect of decoction D3 was very low at all concentrations investigated. The above results were also expressed as the dose required to cause 50% inhibition for each decoction (EC_{50}) and the results are depicted in Table 1. The EC_{50} value for decoction D1, D2 and D3 were 6.8 ± 0.0, 7.3 ± 0.1, 140.9 ± 1.6µg/ml respectively. L-Ascorbic acid was used as the positive standard for antioxidant activity, and the EC_{50} value was 6.4 ± 0.1 µg/ml.

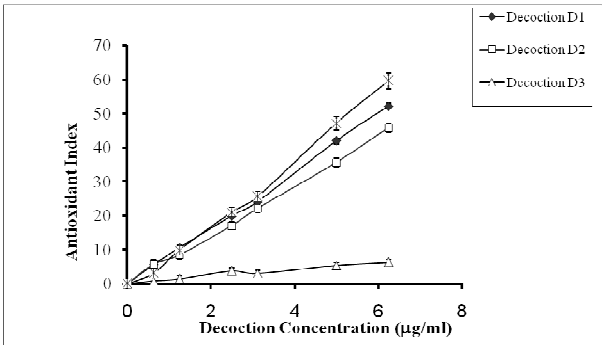

Fig. 1 Free radical scavenging activity of decoctions D1, D2, D3 and L-Ascorbic acid with concentrations using 1,1-diphenyl-2 picryl hydrazyl (DPPH) radical assay. Results are presented as mean +S.E.M. (n=6)

Determination of anti lipid peroxidation activity

The results of the TBARS assay for decoctions D1, D2, D3 and vitamin E are given in Fig. 2. All three decoctions showed antilipid peroxidation activities, which are higher than that of Vitamin E. The percentage antioxidant activity of D1, D2 and D3 increased with increasing concentration as shown in Fig. 2. These results were also expressed as the dose required to obtain 50% antioxidant index and this is given in Table 1.

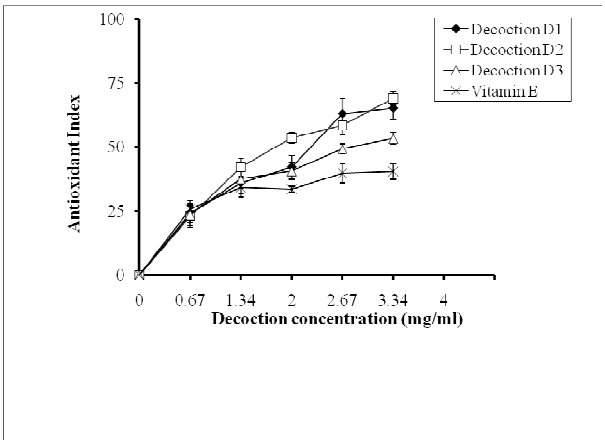

Fig. 2. Antioxidant Index of decoctions D1, D2, D3 and Vitamin E at different concentrations on Lipid peroxidation in egg yolk. Results areresented as mean ± S.E.M. (n=4).

Measurement of reducing power

The reducing power of the water extracts of the three decoctions and ascorbic acid are shown in Fig. 3. Though the reducing power of all three decoctions was found to increase with increasing concentration, the values were remained lower compared to the ascorbic acid. The mean absorbance ± SD of D1, D2, D3 and ascorbic acid at 0.20 mg/ml concentration are 0.45 ± 0.05, 0.40 ± 0.02, 0.04 ± 0.01 and 0.87 ± 0.07 showing that all three decoctions has less activity compared to

L- ascorbic acid. Decoction D3 showed the lowest reducing power at the listed concentration range.

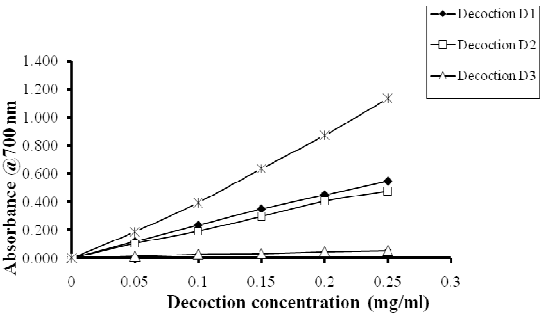

Fig.3. Total reduction capability of different concentrations of decoction D1, D2, D3 and ascorbic acid using spectrophotometric detection of the Fe3+-Fe2+ transformation. Results are presented as mean ± SD (n=6).

Cytotoxicity and cell proliferation assay

Cytotoxic effects, cell viability and proliferation were examined as a preliminary step towards the existence of anti cancer activity in decoction D1, D2 and D3 in RD cells, after treatment with different concentrations (Table 2). LDH release was increased with the concentration in D1 and D2 where as release of LDH with D3 retained at base line level under the concentrations investigated. Decoction D1 is more effective in inducing cytotoxicity and shown 93% of LDH release compared to 68.8% with D2 ($p<0.05$) at concentration of 150 µg/ml. Similarly significant increase ($p<0.05$) in LDH release was observed in other concentrations investigated with D1 compared to D2 except for the concentration of 50 µg/ml.

Cell proliferation studies of RD cells were carried out using MTT assay following 24 hour treatment with D1, D2 and D3. Proliferation was decreased in cells treated with D1 and D2 in a dose dependent manner. Similar to cytotoxicity obtained with LDH assay, D1 was more effective than D2 and 78% of cell survival was shown with D3 even at 200 µg/ml of concentration. (Table 3).

DISCUSSION

A wide array of phenolic substances, particularly those present in dietary and medicinal plants have been reported to posses substantial anticarcinogenic and antimutagenic activities. The majority of these naturally occurring phenolics retain antioxidant anti-inflammatory properties, which appears to contribute to their chemopreventive or chemoprotective activity (31).

The decoction D1 and D2 under the present investigation contains 37% and 30% of total phenolic components where as D3 contains a very low (6%) total phenolic contents. This is not surprising as D1 and D2 have almost similar constituents and having similar phenolic contents may be attributed to the similar plant components present in the two decoctions. A Previous study on a decoction composed of *Terminalia chebula, Terminalia bellerica* and *Phyllanthus emblica* (Thripala), which is very much similar to D1, has been reported that the total phenol content of Thripala was 38% (16). The same study reports that *Terminalia chebula, Terminalia bellerica* and *Phyllanthus emblica* have total phenolic content of 44%, 33%, and 36% respectively. These results are in line with our observations on total phenolic contents of D1 and D2. In addition to the three plants in Thripala, D1 has *Commiphora mukul* and D2 has *Commiphora mukul, Smilax china* and *Nigella sativa*. No previous reports are found on the phenolic content of these three medicinal plants. Their individual contribution on the total phenol content of respective decoction is inconclusive. Also, no reported data was found in phenolic content of decoction D3 or its components.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (25). DPPH radical is usually used as a substrate to evaluate antioxidant activity of substances and our results showed that D1 and D2 to have similar activities. The decoctions D1 and D2 showed EC_{50} values of 6.8 and 7.3 µg/ml respectively. Similar result has been obtained for Triphala where the EC_{50} was 7 µg/ml (16). DPPH radical scavenging activity of individual constituents of *Terminalia chebula, Terminalia belirica* and *Phyllanthus emblica* has been reported and the EC_{50} values are 6, 10, and 8 µg/ml respectively (16). These results indicate that the total free radical scavenging activity of D1 and D2 is mainly attributing from the three aforementioned medicinal plants. DPPH free radical scavenging ability of methonolic extract of *Smilax china* root has been reported by Lee et al. (20). They report that EC_{50} value for methanolic extract of *Smilax china* root was 7.4 µg/ml. *Smilax china* is a component in D2, however this value cannot be directly compared with our results as our study was conducted on the aqueous extract of the decoctions. The decoction D3 has very high EC_{50} value of 140.9 µg/ml. The amounts of total phenolic components are also low in this extract. Hence there seems to have a direct association between total phenolic content and the DPPH free radical scavenging activity.

Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds (27). These include reactive carbonyl compounds. The most abundant among them

is malondialdehyde (MDA), one of the secondary lipid peroxidation products. These carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases (27). Thus the decrease in the MDA levels in the presence of increased concentration of each decoction indicates the role of decoctions as antioxidants. TBARS assay was used to determine the antilipid peroxidation properties of the three decoctions. Egg yolk was used as the lipid rich substrate (26, 27). However, minor change to the procedure had to be adopted as the water extracts of all three decoctions have a colour of dark brown to red which interfered with the resultant colour of malonaldehyde - thiobarbituric acid adduct. Hence a blank for each concentration of every drug was prepared which contained, the sample, thiobarbituric acid, acetic acid and butanol. All the investigated decoctions show protective antioxidant activity at different magnitudes of potency. Vitamin E was used as the positive control in the anti lipid peroxidation assay. According to the results obtained, all three decoctions show high anti lipid peroxidation abilities than vitamin E over the concentration range used. There is no significant difference ($p>0.05$) between decoction D1 and decoction D2 in antilipid peroxidation activity. The high antilipid peroxidation activity of decoctions, D1 and D2 may be attributed to their high phenolic contents. Decoction D3 showed significantly low ($p<0.05$) anti lipid peroxidation activity compared to D1 and D2, under the concentrations investigated.

Anti lipid peroxidation activity of some individual constituents of decoction D1, D2 and D3 has been reported. A dose dependent relationship was observed when aqueous acetone extracts of *Terminalia chebula, Terminalia bellerica*, and *Phyllanthus emblica* were tested for their ability to reduce lipid peroxides generated by $Fe2^{+}$ Ascorbic acid system on mice liver homogenate (17). A study carried out by Naik et al. (16) shows that, Triphala and its major constituents inhibit γ-radiation induced damage in microsomal lipids. Lee et al., (20) studied the anti lipid peroxidation ability of methanol extract of *Smilax china* root, which was one of the constituent of decoction D2. This study was conducted on H_2O_2 treated V79-4 cells where the IC_{50} value was found to be >100 mg/ml. Effect of *Nigella sativa* essential oils, (Thymoquinone, carvacrol and 4- terpineol) on lipid peroxidation of bovine brain extract liposomes has also been reported (19, 21). Since they have used different lipid rich substrates and peroxide radical inducing agents such as ascorbic acid, H_2O_2 and Fe^{2+}ions, it is difficult to make a direct comparison between our results and results found in literature. However, they provide supportive evidences to our results on antilipid peroxidation ability of the drugs present in the decoctions investigated.

For the measurement of the reducing ability, the Fe^{3+}-Fe^{3+} transformation was investigated in the presence of water extracts of each decoction. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (32). Similar to their antioxidant activities, the reducing power of each decoction increased with increasing dosage. L Ascorbic acid showed remarkably higher reducing power than the all three decoctions investigated. The decoction D3 showed very low reducing power as well as low antioxidant activity, which can be associated with its low phenol content.

In addition to antioxidant and anti lipid peroxidation activities of decoction D1, D2 and D3, cytotoxicity studies were carried out using LDH and MTT assays. Quantitative analysis of lactate dehydrogenase (LDH) release is based on the fact that LDH is a strictly cytoplasmic enzyme and the elevation of its level in the culture medium reflects the disruption of the host cell plasma membrane (33). Recent studies suggest that LDH is a more reliable and accurate marker of cytotoxicity, because damaged cells are fragmented completely during the course of prolonged incubation with substances (34). The MTT cell proliferation assay measures the cell proliferation rate. This test is based on the conversion of tetrazolium salts into coloured product, formazan, by the mitochondrial enzyme succinate dehydrogenase. Because only metabolically active cells cleave tetrazolium salts, the number of surviving cells is directly proportional to the level of the formazan product created.

The release of LDH was increased in a dose dependent manner after treatment of decoction D1 and D2 over a period of 24 hours, however for Decoction D3, the release of LDH retained at base line level over the concentrations investigated. Parallel results were obtained for MTT assay. In comparison to the results of LDH and MTT assays, it is observed that the results of both assays are in conformity with each other and associate with phenol content of the relevant decoctions. Further LDH and MTT assays reveal that, these decoctions not only inhibited the proliferation of RD cells but also induced cell death.

Agents capable of inducing apoptosis, inhibiting cell proliferation, or modulating signal transduction are

currently used for the treatment of cancer (35). A combination of multiple chemopreventive agents or agents with multiple targets is considered to be more effective than a single agent (35, 36). Kaur and coworkers (37) have identified gallic acid present in Triphala plays an important role in inducing cytotoxicity and apoptosis in cancer cell lines. Deep et al. (38) reported that Triphala inhibited the induction of benzo(a)pyrene induced fore stomach tumoigenesis and such inhibition may be related to the suppression of cell proliferation and the induction of apoptosis. Among individual ingredients of the decoctions investigated, *Terminalia chebula, Phyllanthus emblica, Commiphora mukul*, *Smilax china* and *Nigella sativa, which* present in decoction, D1 and D2 were reported to have anticancer effects on cancer cells (15, 22, 23, 39). Further Khan et al. (40) revealed that main compound responsible for antiproliferative activity of *Phyllanthus emblica* is Phyrogallol. Samudio et al. (22) have reported antileukemic effects of three steroids, cis-gugulstterone, trans-gugulsteron, and 16-dehydroprogesteron, which are some active components present in the gum resin of *commiphora mukul*. Our study shows that there is a link between *in vitro* cytotoxicity and the total phenolic content of the three decoctions D1, D2 and D3. Some constituents such as *Rubia cordifolia, Picrorhiza kurroa, Azadirachta indica,* and *Perocarpus santalinus* in decoction D3 have been studied for their anticancer properties (41-43). However, to our knowledge no study has been done on the synergistic effect of the components in the decoction D3 towards their anticancer and antioxidant activities.

As a conclusion, it can be stated that the results obtained from the present study clearly showed decoction D1 and D2 had strong and effective antioxidant antiproliferative and cytotoxic activities

Table 1: *Extraction yield, Total phenolic content, DPPH radical scavenging activity and anti lipid peroxidatin activity of decoction D1, D2, D3 and Standard compounds.*

Decoction	Extraction yield (mg/g dry matter)	Total phenol content (%w/wgallicacid equivalents)*	DPPHradical scavenging activity EC_{50} (µg/ml)*	Antilipid peroxidation activity. (TBARS assay) EC_{50} (mg/ml)**
Decoction D1	250	37.5 ± 1.4	6.8 ± 0.0	2.2 ± 0.2
Decoction D2	200	30.5 ± 0.7	7.3 ± 0.1	2.2 ± 0.1
Decoction D3	84	6.4 ± 0.3	140.9 ± 1.6	3.0 ±0.1
Ascorbic acid	-	-	6.4 ± 0.1	-
Vitamin E	-	-	-	4.0 ± 0.1

Data represented as the mean ± S.E.M (n=6) ; Data represented as the mean ± S.E.M (n=4); EC_{50} value was defined as the concentration of 50% inhibition of respective radical

Table 2 : Comparison of dose dependent LDH leakage in RD cells after exposure to decoctions D1, D2 and D3 for 24 h. Data are presented as percentage LDH released to that of control ±SEM (n=3).

Decoction	**Concentration . µg/ml)**	**% LDH release**
Decoction D1	0	0
	50	2.4 ± 0.7
	100	72.4 ± 7.4
	150	93.4 ± 0.5
	200	95.3 ± 0.6
Decoction D2	0	0
	50	2.6 ± 0.9
	100	19.2 ± 4.2
	150	68.8 ± 2.3
	200	88.6 ± 4.2

Decoction D3	0	-
	50	-
	100	-
	150	-
	200	-

- No LDH release compared to the negative controls

Table 3. Dose dependent effects of decoctions D1, D2 and D3 on cell viability of RD cell line. Viability was determined by MTT assay following exposure to decoctions for 24 h. Data are presented as mean of percentage viable cells to that of control in three independent experiments.

.Decoction	Concentration (µgml)	% Cell viability± S.E.M.
Decoction D1	0	100.0
	50	90.4 ± 0.1
	100	29.0 ± 0.4
	150	10.3 ± 1.6
	200	3.2 ± 1.0
Decoction D2	0	100.0
	50	90.5 ± 0.3
	100	73.0 ± 0.7
	150	41.5 ± 0.5
	200	10.1 ± 2.4
Decoction D3	0	100.0
	50	88.6 ± 4.1
	100	82.2 ± 4.7
	150	82.1 ± 4.4
	200	78.9 ± 2.8

compared to the decoction D3. Also it can be inferred that above activities were directly correlate to the total amount of phenolics found in each decoction. The additive roles of phytochemicals may contribute significantly to the potent antioxidant activity and the ability to inhibit cancer cell proliferation *in vitro*. Since this is a preliminary study on the anticancer potential of above selected decoctions, further chemical and pharmacological work at molecular level are required to establish the possible correlation among the investigated activities of the above herbal preparations.

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