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FINAL REPORT

Induction of Apoptosis in Cancer cell lines by Selected Traditional Medicines

Principle Investigator:

Dr. S.S.S.B.D.P.SOYSA

NSF registration Number: **RG/2005/HS/17**

FR 1715

Contents

Section 1

Information regarding the Project/Project Personal: Grant number RG/2005/HS/17

I. Contact Number: 2697485

II. **Title of the project:** Induction of Apoptosis in Cancer cell lines by Selected Traditional Medicines

III. **Principal Investigator**: Dr.S.S.S. B.D.P. Soysa
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IV. **Co-investigators:** Mr. M.G.A.N Perera,
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V. Institute where Research is being carried out,

Department of Biochemistry and Molecular Biology,
Faculty of medicine,
Colombo **08**

VI. Date of Award: 22.08.2005
VII. Date of completion of Project: July 2010
VIII. Total allocation of funds (Rs): 1,862,250.00
IX. Total spent (Rs): 1,745,992.75
X. Number of Students employed:
Seven part time research students as a partial fulfillment of their postgraduate degrees

Students' informations

Five postgraduate students and one undergraduate student were involved under this research theme as a partial requirement for their degrees. One student registered for M.Phil degree is still working for her degree.

i. Names of Research Students (2006/2007)
Mr. M.G.A.N. Perera- M.Sc in Analytical Chemistry

ii. Priyantha Ihalagamage - (2007/2008)
M.Sc in Bio Chemistry and Molecular Biology

iii. A.A. Subashini Adikary -2007 (Not Completed to date)
M.Phil Student

iv. Kalhari Silva (2008/2010)
M.Sc in Bio Chemistry and Molecular Biology

v. Irushi De Silva (2008/2010)
M.Sc in Bio Chemistry and Molecular Biology

vi. Sahani Weerasekara-2008/2009
B.Sc in Parmacy

vii. (Inoka Manikpurage) - Part of her M.Phil study

XI. Post graduate degree completed with dates

1. Mr. M.G.A_N Perera- M.Sc in Analytical Chemistry (2007)

Induction of Apoptosis in Cancer cell lines by Selected Traditional Medicines

2. Priyantha Ihalagamage - (2007/2008)
M.Sc in Bio Chemistry and Molecular Biology
Apoptosis and Cytotoxic Studies with Selected Traditional Medicinal Preparations

On pending results- Completed the research component as a partial requirement for the degree

3. L.I. Kalhari Silva – (2008/201) M.Sc in Bio Chemistry and Molecular Biology

Antioxidant and cytotoxic activities of a decoction prepared from ***Adenanthera pavonina*** **and** *Thespesia populnea*

4. Irush De Silva – (2008/2010) M.Sc in Bio Chemistry and Molecular Biology

Antioxidant and cytotoxic activities of a decoction prepared from *Fleuggea leucopyrus Wild (Leaves)*

5 (Inoka Manikpurage) - Part of her M.Phil study
Cytotoxicity of *P. cystidiosus* mushroom extracts/ fractions

XII. Number of Technical Assistants: One (for one year).

Section 2

Executive Summary of the Project:

Traditional remedies have a long-standing history in Sri Lanka and still popular for treating many ailments including cancer. Induction of apoptosis is a cancer preventive process and related studies were carried out with six prescriptions provided by traditional doctors. Decoction 1 (*Terminalia bellerica, Terminalia chebula, Phyllanthus emblica* and *Commiphora mukul*), Decoction 2 (*Terminalia bellerica, Terminalia chebula, Phyllanthus emblica, Commiphora mukul, Smilax china* and *Nigella sativa*), Decoction 3 (*Munronia Pumila, Azadirachta indica, Solanum surattense, Solanum xanthocarpum, Rubia cordifolia, Picrorhiza kurroa , Trichosanthes cucumerin* and *Pterocarpus santalinus*), Decoction 4 (*Boerhavia diffusa, Toddalia asiatica, Anacyclus pyrethrum, Ricinus communis, Crateva adensonii, Bombax ceiba*), Decoction 5 (*Adenanthera pavonina, Adenanthera pavonina. L*), Decoction 6 (*Flueggea leucopyrus*) and `pranajeewa oil' are the herbal drugs used in this study. Antioxidant activity, phenolic and gallic acid were determined for all decoctions. RD or *HEp* 2 cell lines were treated for 24 hours with respective drug and cytotoxicity was determined by LDH, MTT assays and protein synthesis. Morphological changes were determined by light microscopy and fluorescent microscopy.

Decoction 1 showed the highest phenolic and gallic acid contents. The antioxidant activity and phenolic content is in order of D1>D2≈D5>D6>D4. *Bombax ceiba ,Anacyclus pyrethrum* are two other herbs which showed high phenolic content and antioxidant activity. The EC50 values for DPPH scavenging activity were very low with D1, D2 and D5 and comparable to the ascorbic acid. The phenolic content was exponentially correlated [$y = 1896.3x^{-1.5149}$ ($r^2 = 0.7719$)] with the EC50 for DPPH radical scavenging activity. The mean EC50 for LDH assay for D1, D3, D4, D5 and D6 were 94.4, 129.7, 1060, 178.8 and 254.5µg/ml respectively. It is observed that the EC50 values for both LDH and MTT assays were linearly correlated ($y = 1.192x - 37.56$ $R^2 = 0.999$) each other, and exponentially correlated ($R^2 > 0.96$) with the phenol content of the decoctions. The morphological changes were appeared gradually with increasing concentration at different levels for each decoction. The results obtained for LDH, MTT and protein synthesis were compatible with the morphological changes observed for each decoction. The morphological changes show that the cell death induced by decoctions is mediated through apoptosis.

Among the six decoctions investigated, D1, D2, D5 and D6 are very effective in cytotoxicty and polyphenols play an important role in this regard.

Section 3

3.1 Introduction

Traditional remedies have a long-standing history in Sri Lanka and still popular for treating many ailments. Many plant derived chemicals are the basis of conventional drug therapies. Over 3000 species of plants have been reported to have anticancer properties (Graham *et.al*, 2000).

Some of the screening tests have been carried out on the basis of apoptosis process or a programmed cell death. It is a highly regulated process that involves the activation of a series of molecular events, leading to cell death. Apoptosis is characterized by cellular morphological change such as membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of cell debris by neighbouring cells (Elmore, 2007). It plays an important role as a protective mechanism in the organism by removing damaged cells or over-proliferating cells due to an improper mitotic stimulus (Wyllie, 1999). It is therefore possible to take advantage of this intrinsic mechanism by manipulating the apoptotic process for therapeutic gains in cancer treatment.

Reactive oxygen species (ROS) are constantly being produced in animal cells and associated with DNA damages , inducing premutagenic modifications of nucleotides and promoting oxidation of proteins and lipids.. Data support that increased formation of ROS may play an important role in carcinogenesis, atherosclerosis, diabetes, emphysema, cataracts, and neurodegenerative diseases (Gago-Dominguez, 2005). ROS seem to be mediators or triggers of protective mechanisms, such as apoptosis, phagocytosis, and detoxification reactions.

The traditional drugs used for cancer treatments in Sri Lanka are made of decoctions comprising several medicinal plants or a single plant. Only countable studies on cytotoxicity have been reported so far regarding traditional preparations of drugs used in Sri Lanka for cancer treatment. We initiated investigations for the first time in Sri Lanka to screen and study the cytotoxic properties of drugs currently used in cancer treatment. In addition to decoctions investigated, different types of Sri Lankan tea products also investigated for cytotoxicity using cancer cell lines.

3.2 Objectives

Objectives of this study were to:

(1) evaluate and compare the total antioxidant capacity by common antioxidant activity methods, and phenolic content

(2) identify and quantify gallic acid present by RP-HPLC;

(3) determine the relationship between antioxidant activity and phenolic compounds

(4) Cytotoxic activity and capacity to induce apoptosis
of selected decoctions which is used for cancer therapy in Sri Lanka

3.3 Chemicals and Equipment

3.3.1. Reagents and Chemicals

The chemicals, Gallic acid, Folin ciocalteu reagent, Acetinitrile (HPLC grade), β-hydroxyethyltheophylline, Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), antibiotics (penicillinase/ Streptomycin), Trypsin (from porcine pancreas), Ethylenediamine tetra acetic acid (EDTA), 3,4,5-(dimethylthiazol-2-yl)2-5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA), Trizma hydrochloride (Tris [hydroxymethyl]-aminomethanehydrochloride), Lauryl sulfate (sodium dodecyl sulphate), Sodium chloride, phenol, Agarose gel, Ethidium bromide, Campothecin, Chloroform and Isoamyl alcohol were purchased from Sigma chemicals Co. (St. Louis, MO 63178 USA).

L-glutamine, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Polyethylene glycol tert – octylphenyl ether (Triton X-100) solution (1%), Cycloheximide solution, Dimethyl sulfoxide and RNaseA were purchased from Fluka (Fluka chemie GmbH, CH – 9471 Buchs). The chemicals Ascorbic acid and Ethanol were purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England) and Proteinase k from promega (2800, Woods Hollow Rd., Madison, WI 53711- 5399, USA). All the chemicals used; Sodium bicarbonate, Sodium chloride, di-Sodium hydrogen phosphate, Potassium di-hydrogen phosphate, Potassium chloride, Sodium potassium tartrate, Sodium carbonate, Sodium hydroxide, copper sulphate ($CuSO_4\,5H_2O$) Isopropyl alcohol, sulfanilamide, N-(1-naphthyl) Ethylenediamine- dihydrochloride, Thiobarbituric acid, Butan-1-Ol and Acetic acid; were in analytical grade.

The Lactate Dehydrogenase (LDH) enzyme assay kits were purchased from Roche (Roche diagnostics GmbH, Germany) and Randox (Randox Laboratories Ltd., Crumlin Co. Antrim, UK). The Caspase colorimetric assay kit was purchased from Biovision (Biovision Research products, CA 94043, USA).

3.3.2. Equipment

LABCONCO Freeze Zone 2.5 (LABCONCO corporation, Kansas city Missouri 64132 – 2696) freeze dryer was used to get the decoctions freeze dried (at -40°C and vacuum range was 0.22- 0.01 mBar). SHIMADZU UV 1601 UV - visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used to read absorbance. Centrifugation of samples and cell suspensions were carried out in Kubota 6500 (Kubota Corporation, Tokyo, Japan) high speed refrigerated centrifuge and in Biofuge D - 37520 (Heraeus instruments) centrifuge. SHIMADZU AEG-220 analytical balance (Shimadzu corporation, Kyoto, Japan) was used to prepare standard solutions and the balance, METTLER PJ 6000 (Mettler instrumente AG, Switzerland) used for preparation of buffers and other solutions. SHIMADZU High Performance Liquid Chromatographic system equipped with LC10AS solvent delivery system, SPD-10AVP UV visible detector, DGU-14A degasser and Chromatopac CR8A printer was used to determine the Gallic acid content. Gallenkamp Spinmix (Gallenkamp service organization, Wesy, Sussex, RH 102RE UK) vortex mixer was used for mixing. Cells

were incubated at 37^{0}C at humidified CO_2 incubator (SHEL LAB / Sheldon manufacturing Inc. Cornelius, OR 97113. USA) and cells were observed using Olympus (1X70-S1F2) inverted fluorescence microscope (Olympus Optical Co. Ltd. Japan). The cell counts were taken using Neubauer haemocytometer (Bright-Line/Neubauer from Assistant – Germany). ESCO (EQU / 04 – EHC) horizontal laminar flow cabinet (EBSCO Micro pte. Ltd., Singapore 486777) was used to carry out all cell culture experiments. Water baths Sshutzart. (DIN 40050 / Sshutzart, Germany) and Memmert (Memmert GmbH Co. KG, Schwabach, FR of Germany) were used for heating. The pH meter (pH cube / TPS pty. Ltd.) was used to measure the pH value of buffer solutions.

For all experiments ultra filtered water from LABCONCO (waterproplus) UV ultra filtered water system (LABCONCO Corporation, Kansas city Missouri 64132 – 2696) or distilled water was used.

3.3.3 Plants and Materials

Following plant materials were used in preparation of decoctions and investigated for phenolic content, antotoxidant activity and cytotoxicity.

1. **Decoction 1**- Consisted of 4 herbal components namely, *Terminalia bellerica* (fruit), *Terminalia chebula* (fruit), *Phyllanthus emblica* (fruit) and detoxified *Commiphora mukul* (resin)

2. **Decoction 2**- Consisted of 6 herbal components namely, *(Terminalia bellerica* (fruit), *Terminalia chebula*(fruit), *Phyllanthus emblica*(fruit), *detoxified Commiphora mukul*(resin), *Smilax china* (rhizome) and *Nigella sativa* (seeds)

3. **Decoction 3** - Consisted of 8 herbal components *Munronia Pumila* (leaves), *Azadirachta indica* (bark), *Solanum surattense* (root), *Solanum xanthocarpum* (whole plant), *Rubia cordifolia* (whole plant), *Picrorhiza kurroa* (root), *Trichosanthes cucumerin* (leaves) and *Pterocarpus santalinus* (heart wood)

4. **Decoction 4** - *Boerhavia diffusa* (Root), *Toddalia asiatica* (Root), *Anacyclus pyrethrum* (Root), *Ricinus communis* (Root), *Crateva adensonii* (Bark), *Bombax ceiba* (Gum).

5. **Decoction 5**- *Adenanthera pavonina* (bark) , *Adenanthera pavonina. L* (bark)

6. **Decoction 6** - *Fluegged leucopyrus* (Leaves)
   All ingredients were in dried form.

7. Considering the national importance and the beneficial health properties of tea (*Camellia Sinensis*), Black tea, Green tea and White tea manufactured in Sri Lanka were studied for its possible cancer preventive activity.

8. Pranajeewa oil

9. *Studies on antioxidant activity and Cytotoxicity on various fractions of *Pleurotus cystidiosus*

*This study was carried out for a request made by Prof. DTU Abeythunga

Gallic acid (GA) and phenolic content were quantified and antioxidant activity was determined in following plant materials

1. Imbul Malliyam – *Bombax ceiba*
2. Cheena ala - *Smilax china.*
3. Akkarapatta - *Anacyclus pyrethrum*
4. Iramusu - *Hemidesmus indicus*
5. Kabressa - *Smilax zeylanica* (GA was not quantified)

3.3.4 Collection of herbal materials

All the dried herbs were obtained from Bandaranayake Memorial Ayurveda Research Institute and from a registered Ayurvedic drug outlet at Colombo which are registered in Ayurveda Drug Cooperation in Sri Lanka. 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.

3.3.5 Identification of plant materials

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.

3.3.5 Sources which obtained the prescriptions of decotions

Dr.S.Senarathne, Gampaha (Decoction 1, 2 and 3), Dr. Tikiri Bandara, Kurunegala (decoction 4), Dr. Nimal Jayathilake, Brandigampola Wedagedara, Labugama Road, Hanwella (decoction 5). The traditional herbal oil, 'Pranajeewa' was kindly donated by Dr. S Vithana Sethsuva Ayurveda Hospital, Batalanda Road, Makola South, Sri Lanka

3.4 Experimental

3.4.1 Preparation of Decoctions

Drug	Plant material	Common name	Weight (g)
Decoction 1	*Terminalia bellerica* (fruit)	Bulu	15
	Terminalia chebula (fruit),	Aralu	15
	Phyllanthus emblica (fruit)	Nelli	15
	Commiphora mukul (resin)	Gugul	15
Decoction 2	*Smilax china* (rhizome)	Cheena Ala	7.5
	Nigella sativa (seeds)	Kaluduru	7.5
	Terminalia bellerica (fruit	Bulu	7.5
	Terminalia chebula (fruit),	Aralu	7.5
	Phyllanthus emblica (fruit)	Nelli	7.5
	Commiphora mukul (resin)	Gugul	7.5
Decoction 3	*Munronia Pumila* (leaves),	Binkohomba	10
	Azadirachta indica (bark),	Kohomba	10
	Solanum surattense (root),	Ela-Batu	10
	Rubia cordifolia (whole plant),	Wel Madata	10
	Solanum xanthocarpum (whole plant),	Katuwelbatu	10
	Picrorhiza kurroa (root),	Katukarosana	10
	Trichosanthes cucumerin (leaves)	Dummella	10
	Pterocarpus santalinus	Rath Handun	10
Decoction 4	*Boerhavia difusa (Root)*	Pitasudu Sarana	8.3
	Toddalia asiatica (Root)	Kudu Mirissa	8.3
	Anacyclus pyrethrum (Root)	Akkarapatta	8.3
	Ricinus communis (Root)	Endaru	8.3
	Crateva adensonii (Bark)	Lunuwarana	8.3
	Bombax ceiba (Gum)	Ela Imbul	8.3
Decoction 5	*Adenanthera pavonina*	Madatiya	30
	Thespesia populnea	Gansurya	30
Decoction 6	*Fleuggea leucopyrus Wild (Leaves)*	Katupila	250g/l

Decoctions 1 to Decoction 5 mentioned above were prepared according to the procedure practiced by the traditional doctors in Sri Lanka. Equal amount of dried herbal components present in each decoction as described in the table were mixed and boiled with 1400 ml of water until the volume get reduced up to 175 ml ($1/8^{th}$ of the original volume). Clay pot with a lid was used to boil the contents. The leaf powder of D6 was extracted in distilled water

(250g/L) by refluxing for 2-3 hours. 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°C until used.

3.4.2 Cell cultures

Rhabdomyosarcoma (RD) and laryngeal epithelial carcinoma *(HEp 2)* cell lines were used to determine cytotoxicity. The cell lines were obtained from Medical Research Institute, Colombo.

3.4.3 Growth Media

Cells were cultured in EMEM or RPMI growth media supplemented with 10% fetal bovine serum (FBS), MEM non essential (1%), L-glutamine (3%), 50 IU / mL penicillin and 50 µg / mL streptomycin. The pH of the growth media has been adjusted to physiological pH (7.4) using 7.5% sodium bicarbonate. Cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity.

3.4.5 Phytochemical analysis

3.4.5.1 Determination of total phenolic content

Total Phenolic contents were determined using the Folin- Ciocalteu method (Makkar *et al.*, 1993). Briefly, 50 µl of the water extract of each drug was diluted with 450 µl of distilled water and 250 µl 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.

3.4.5.2. Determination of Gallic acid content by High performance liquid chromatographic (HPLC)

The HPLC method developed in the Department of Biochemistry and Molecular Biology was used to determine the Gallic acid content in decoctions (unpublished data). The mobile phase constituted acetinitrile (8 %) in acetic acid (1%) at a flow rate of 1.2mL/min. The internal standard was β-hydroxyethyl-theophilline (100µg /mL). The separation was performed on a C 18 column (3.9 x 300mm) and a guard column. The eluents were detected at 260nm. Different concentrations of decoctions (2.5, 5, 10 mg/ml) were prepared from the stock solution (10mg/mL) to quantify gallic acid content. The calibration curve was constructed

using Gallic acid (2, 10, 20, 30, 40, 50 µg/mL) prepared in ultra pure water to determine the Gallic acid content present in decoctions.

3.4.6 Antioxidant activity

3.4.6.1 DPPH radical scavenging activity

This method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 515 nm.. Free radical scavenging activity of the decoctions was assayed by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) scavenging method described by Blois (1958) with slight modifications. Stock solutions (10mg/ml) were diluted with water to obtain required optimum concentrations. A volume of 50 µl of the sample was mixed with 950 µl of DPPH (100 µM) in absolute ethanol. Mixture was allowed to stand for 30 minutes in dark at room temperature. Deionized water (50 µl) was used as the control. The absorbance (A) was measured at 515 nm compared with the control (Maximum absorbance). Ascorbic acid was used as the standard antioxidant. The scavenging activity of samples was correlated to the intensity of quenching DPPH. The results were expressed as percentage anti-oxidant index (AI %) using this equation, AI = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. 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.

3.4.6.2 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 (Baratta *et al.*, 1998 and Kulišić *et al.*, 2006). Briefly, 100 µl of egg yolk (10% w/v) in KCl (1.15 %) and 50 µl of sample prepared in water 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 dedocyl 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 (Kulišić *et al.*, 2006).

3.4.6.3 Measurement of reducing power

The reducing power of each decoction was determined according to the method used by Dhalwal *et al.* (2005) with slight modifications. Different concentrations of decoctions (100

µl) were mixed with 250 µl of phosphate buffer (0.2 M, pH 6.6) and 250 µl of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min, and 250 l 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 $FeCl_3$ (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.

3.4.7 Cell culture and Cytotoxicity and cell viability assays

Rhabdomyosarcoma cells were seeded in 12 well plates (NUNC, Denmark) at a density of 2x 10^5 cells /well and cultured overnight for the decoctions D1, D2 and D3. *HEp 2* cells were seeded in 12 well plates (NUNC, Denmark) at a density of 2 x 10^5 cells/well and cultured overnight for the decoction D4. *HEp 2* cells were seeded in 24 well plates (NUNC, Denmark) at a density of 1 x 10^5 - 2 x 10^5 cells/well for the *Pleurotus cystidiosus* and pranajaeewa oil. In all experiments a negative control and a positive control were maintained. Negative control contained only growth media while the positive control contained campothesin (5mM, 20µl).

3.4.7.1 Determination of cytotoxicity by lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme which is released from intact cells in cell damage can be used as an indicator of cell viability. Leakage of LDH was examined after exposure of drugs to cell lines. Cells were seeded in 12 or 24 well plates as previously described and cultured overnight. Confluent monolayers were treated with different concentrations of decoction D1, D2, D3 and D4, and incubated in CO_2 incubator at 37°C for 24 hours. LDH activity in the supernatant and the cell lysate were measured using the LDH cytotoxcity detection kit (ROCHE diagnostics, Mannheim, Germany) following the manufacturer's instructions. The cells were treated with 0.1% Triton X-100 to obtain cell lysate. The amount of LDH released to the medium by each decoction was indicated as a proportion of the total LDH activity i.e the LDH activity of the supernatant and the lysate. 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.

3.4.7.2 Determination of cell viability by MTT assay

The MTT assay (Fotakis and Timbrell, 2006; Plumb, 2004) is based on the ability of metabolically active cells to reduce MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to blue formazan compounds by mitochondrial dehydrogenases. *RD* and *HEp 2* cells were cultured overnight at a density 1 x 10^5 -2 x 10^5 cells/well as previously described. Confluent monolayers were treated with different concentrations of decoction D1, D2, D3 and D4 prepared in media. The cells were

incubated in humidified CO_2 incubator at 37°C for 24 hours. Then the media was replaced by new media (2 ml or 0.5 ml*) and 200 µl or 50 µl* of MTT (5 mg/ml) filtered through the syringe filters (0.45µm) was added to each well. Cells were then incubated at 37°C for another 4h in dark. The medium was aspirated carefully and the formazan product was solubilized with 2 ml or 700 µl* of 0.05 M HCl in 2-propanol. Absorbance was measured at 570 nm.

The percentage cell viability was calculated using the following equation (Fotakis and Timbrell, 2006).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of the test sample x 100 \%}}{\text{Absorbance of the negative control}}$$

* The volumes used for 24 well plates

3.4.7.3 Determination of Total Cell Protein Content

The effects of decoctions on protein synthesis was assayed by Sulforhodamine B Assay (Skehan, 1985) or Hartree – Lowry assay (Hartree *et al*, 1972) method.

3.4.7.3.I Sulforhodamine B Assay

The Sulforhodamine B (SRB) assay, is based on the ability of the SRB dye to bind electrostatically to the basic amino acid residues (Skehan, 1985). Briefly, 24-well plates were seeded with cells (2×10^5) in the culture medium (1ml) and allowed to grow to at least 70% confluence as described elswhere. The wells were then treated with different concentrations (40, 60, 80, 90, 100, 110, 120, 130, 140, 160µg/ml) of the decoction 5 and cultured overnight in 24-well plates in a humidified CO_2 incubator. The cell supernatant was then completed removed and trichloroacetic acid (TCA) (10%: 500µl) was added to fix the cellular protein and the plate was incubated at 4°C for at least one hour prior to the SRB assay. The plate was then washed with five washing cycles using deionised water and dried completely. A volume of 500µl of SRB (0.4% SRB dissolved in 1% CH_3COOH) was added to each well and allowed to stain for a 30 minutes. The stain was removed and the plate was subjected to five washing cycles again to remove unbound dye using 1% acetic acid (vol/vol). After air-drying, the protein bound dye was solubilized with Tris base (10mM: 500µl) and the plates were shaken for at least 30 minutes to homogenize the dye solution. The absorbance was then recorded at 564nm using Tris base as blank. The percentage viability was calculated as given below:

$$\text{The Percentage Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.4.7.3. II Protein Estimation

Proteins present in the cell lysate were determined to study the effect of the drug on protein synthesis by the modified method of Hartree – Lowry assay (Hartree *et al.* 1972). The cells in each well were harvested with 2 ml of Triton X-100 after exposure to different concentrations of D4 and D6 over 24 hours. Contents were sonicated for 20 seconds and centrifuged at 4000 rpm for 5 minutes to obtain the cell lysate. A volume of 1 ml was used to determine the protein content.. The blank was prepared by the addition of 1 mL of deionized water. Bovine serum albumin (BSA) was used as the standard.

3. 4.7. 4 Analysis of cytotoxic activity using Brine shrimp bioassay

Brine shrimp (*Artemia salina*) lethality bioassay is widely used in the bioassay for the bioactive compounds (Meyer *et al.*, 1982) and used to investigate the cytotoxicity of `Pranajeewa` oil` , D5 and D6. The eggs of the brine shrimp were purchased from an aquarium shop, Colombo, Sri Lanka.

3. 4.7.4.1 Preparation of hatching medium

Sea water was collected into plastic containers from the sea (Galle Face Green, Colombo, Sri Lanka). It was heated at 80 ^{o}C for one hour in a water bath and filtered through Whatmann filter paper (No 1). The pH of the resulting sea water which acts as the hatching medium was checked using a pH meter. The sea water used in the experiment, had a pH value between 8.0-8.5. The prepared sea water was stored in plastic bottles and stored in the refrigerator (4 ^{o}C). The prepared sea water was used within two months.

3. 4.7.4.2 Hatching the Brine shrimp

Brine shrimp eggs were hatched in a Petri dish (8 cm. diameter) filled with hatching medium (once taken from the refrigerator the hatching medium was aerated and allowed to come to the room temperature before use). A plastic strip with several 4 mm holes were clamped inside the dish to make two compartments. The eggs were sprinkled on to one compartment, which was darkened by covering with black paper. The larger compartment was illuminated. After 48 hours the photophillic active napulii, free from their egg shells were collected from the illuminated side using a micro pipette for the assay(Meyer *et al.*, 1982) .

3. 4.7.4.3 Bioassay procedure

Stock solution of herbal oil was prepared by dissolving 1 ml of oil in 10 ml of 95 % ethanol (100µl/ml). Different concentrations of herbal oil was prepared by diluting the working standard with ethanol (95%) to get different concentrations of 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20 µl/ml of oil. A volume of 0.5 ml was added to microwell plates (2.0 ml) and kept at room temperature to evaporate ethanol. Aerated hatching medium (2.0 mL) was then added and 10 shrimps were transferred to each well using a micropipette. Controls (n=3) were also

prepared with 2 ml hatching medium with 10 shrimp in each well. The plates were placed under the illumination at room temperature (25-28 °C), and after 24 hours the number of survivors was counted. Any shrimp was moving but not making forward progress was counted as dead. Live healthy larvae were in constant motion (Dhalwal, 2005).

Percent death at each concentration was determined using the formula given below.

$$\% \text{ death} = [(\text{Number of deaths in test} - \text{Number of deaths in control}) * 100] / \text{Number of larvae in control}$$

The dose response curve of % inhibition against concentration was plotted.

3.4.7.4.4 DNA Fragmentation

The *HEp 2* cells were cultured in a 60 mm culture dish at a density of 5×10^5 cells and treated with decoctions for 24 h. The cell pellets were incubated for 60 min at 50 °C in 100 ml lysis buffer (100mM Tris–HCl pH 8, 100mM NaCl and 10mM EDTA). Proteinase K (10µl of 20 mg/ml) was added and further incubated for 30 min at 50 °C. RNase (3 ml of 10 mg/ml) was then added and incubated for 2 h at 50 °C. The DNA was extracted with phenol–chloroform--isoamyl alcohol, subjected to 1.8% of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light transilluminator.

3. 4.7.5 Morphological determination

3. 4.7.5.1 Light microscopy

Cells were seeded in 24-well plates (2 x 10^5 cells per well), and left to adhere to the plastic plates overnight as described in earlier. The cells were then exposed to the decoction at different concentrations.. The morphological changes of cells were detected by microscopic examination of cells using Olympus (1X70-S1F2) inverted fluorescence microscope at 100X magnification and photographed using Nikon D700 camera. (Film speed- 1000asa; 105mm macro lens)

3.4.7.5.2 Ethidium Bromide/Acridine Orange Staining (EB/AO staining)

Induction of apoptosis by the decoction 5 and *Fluegged leucopyrus* (D6) were investigated with AO/EB dye staining as described by Ribble *et al.*, (2005). Briefly, 24-well plates were seeded with cells (2 x 10^5) in the culture medium (1ml) and allowed to grow to at least 70% confluence as described earlier. The wells were then treated with different concentrations of the decoction and cultured overnight in a humidified CO_2 incubator. The supernatant was transferred to 15 ml tubes. The rest of the adherent cells were detached with Trypsin-EDTA (1ml) and incubated 37°C for 2 minutes. The supernatant and the detached cells from the same sample were pooled together in the 15 ml tubes. The *HEp*-2 cells were pelleted by centrifuging at 1,000 RPM for 5 minutes. Cell pellets were then re- suspended in 25µl cold

PBS and 2µl EB/AO dye mixture (The dye mixture for the EB/AO staining was 100µg/ml acridine orange and 100µg/ml ethidium bromide in PBS) was added. Stained cell suspension (10µl) were placed on a clean microscope slide and covered with a cover slip. The cellular morphology was evaluated using Olympus (1X70-S1F2) inverted fluorescence microscope at 400X magnification and photographed using Nikon D700 camera. (Film speed- 1000asa; 105mm macro lens)

3.5 Statistical analysis

All experiments were carried out at least in triplicates of separate experiments. The amount of extract needed, to inhibit free radicals concentration by 50% or inhibit cell growth by 50%, (EC_{50}), was graphically determined by using MS- Windows based software. The linear segment of the sigmoid curves were used to determine EC_{50}. The R^2 >0.99 was considered as linear for the relevant calibration curves. Results were expressed as graphically or mean ± standard deviation (SD) unless specified. A value of $P<0.05$ is considered as statistically significant.

3.6 Results

3.6.1 Extraction yield

High variation was observed with the extraction yield between 6.0-250mg/g (Table 1) for the plant materials studied. Highest phenolic content was found in D1 and D5 (Table 1). Gallic acid was detected in 5 decoctions investigated with different formulations, which the highest concentration was observed with D1. Among the four plant materials investigated, gallic acid detected only in *Bombax ceiba* (Imbul maliyam) and *Smilax china* (Cheena ala) and the values were 1.464 and 0.942 mg/g respectively (Table 1). Flavonoid content was studied in Decoction 5 and the value was 41.80 ± 1.31 w/w % of ECGC equivalents.

Table 3.6.1- Extraction yields, total phenolic, and Gallic acid (GA) contents of decoctions and plant materials

Decoction or Plant material	Extraction yield (mg/g)	Total phenol content (%w/w GA equivalents)*	GA content (mg/g)
Decoction D1	250	37.5 ± 1.4	6.97 ± 0.03
Decoction D2	200	30.5 ± 0.7	5.89 ± 0.14
Decoction D3	84	6.4 ± 0.3	1.14 ± 0.00
Decoction D4	53.4± 0.8	14.42 ± 0.67	3.54 ± 0.06
Decoction D5	60.0± 24.83	34.14±3.54	0.58 ± 0.01.
Fleuggea leucopyrus Wild			-
Leaf (D6)	66	22.15±1.65	NA
Stem	110	12.08%	ND
Iramusu	60	14.52 ± 1.13	0.94
Cheena ala	210	19.43 ± 2.89	1.46
Imbul maliyam	220	32.57± 5.04	ND
Akkarapatta	-	30.98±2.97	-
Kabaressa	5.5	6.62 ± 0.50	NA
Pranajeewa Oil		2.63±0.32mg GAE/mL.	

Data represented as the mean ± S.E.M of replicates (n≥3), ND- Not Detected
NA- not available

3.6.2 The DPPH radical scavenging activity, antilipid peroxidation, inhibition of OH radical production and NO scavenging of decoctions and plant extracts

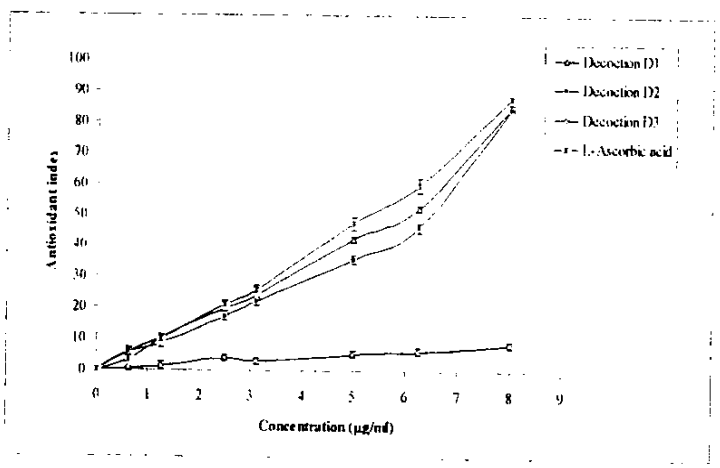
The EC_{50} values in plant extracts used in this study are illustrated in Table 3.5.2. The effect of Decoction 5 and *Fleuggea leucopyrus* (decoction 6) on inhibition of hydroxyl radical production was assessed by the iron(II)–dependent deoxyribose damage assay. The EC_{50}

values were 23.77±3.87µg/ml and 53.21 ± 2.82 µg/ml for *Fleuggea leucopyrus* (D6) and decoction 5 respectively. The EC_{50} values for inhibition of nitric oxide generated from sodiumnitroprasside (SNP) were 4.82±1.82µg and 14.02 ± 0.66 µg/ml for *Fleuggea leucopyrus* and decoction 5 respectively.

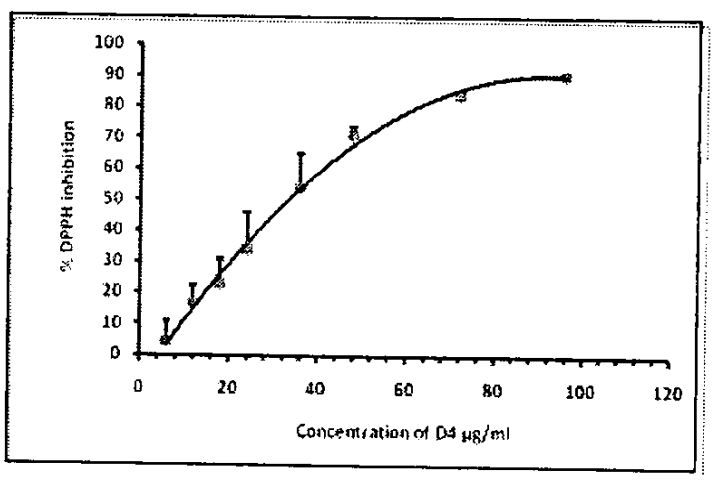
Table 3.6 2. The EC_{50} values (Mean±SD) obtained for DPPH radical scavenging activity, antilipid peroxidation of decoctions and plant materials (n≥3)

	DPPH scavenging activity EC_{50} (µg/ml	Antilipid peroxidation activity.EC_{50} (mg/ml)**
Decoction D1	6.8 ± 0.0*	2.2 ± 0.2**
Decoction D2	7.3 ± 0.1*	2.2 ± 0.1**
Decoction D3	140.9 ± 1.6*	3.0 ±0.1**
Decoction D4	40.18 ± 3.7	-
Decoction D5	7.24 ± 0.50	>5mg/ml
Decoction D6 Leaf Stem	 14.02±0.66 17.556	>5mg/ml
Pranajeewa oil (µl/ml)	79.84 ± 5.35	19.24 ± 0.52
Ascorbic acid	6.4 ± 0.1*	
Vitamin E		4.0 ± 0.1**
Bombax ceiba	15.47±1.80	NA
Cheena ala- *Smilax china*	35.67±0.64	NA
Anacyclus pyrethrum	15.01+0.82	NA
Hemidesmus indicus	46.78±16.03	NA
Smilax zeylanica	121.99 ± 15.86	NA

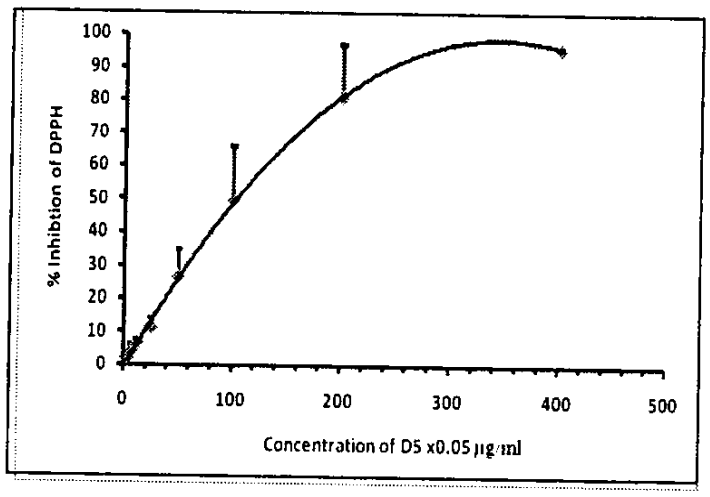
*Data represented as the mean ± S.E.M (n=6). ** Data represented as the mean ± S.E.M (n=4). EC50 value was defined as the concentration of 50% inhibition of respective radical. NA- Not available



(a)



(b)



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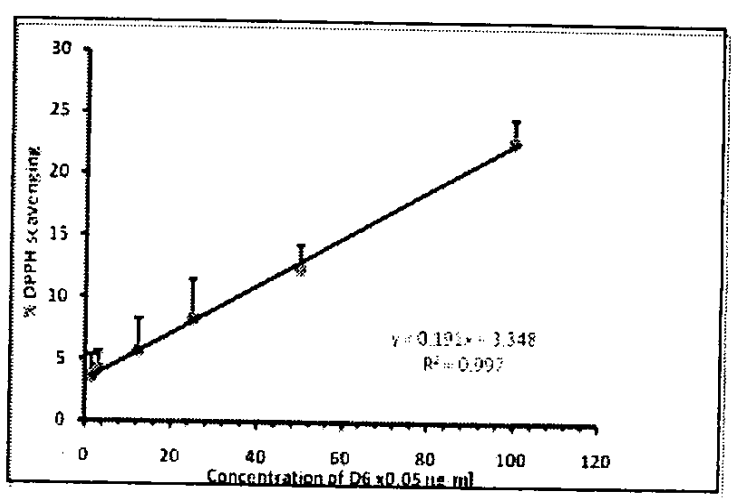
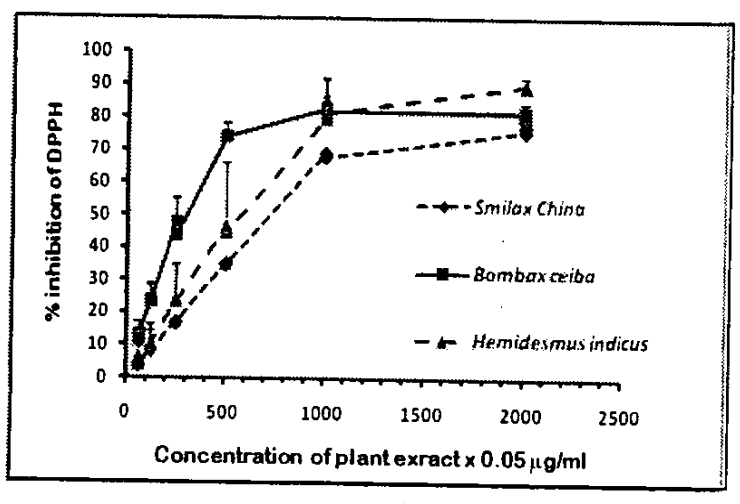


(d)



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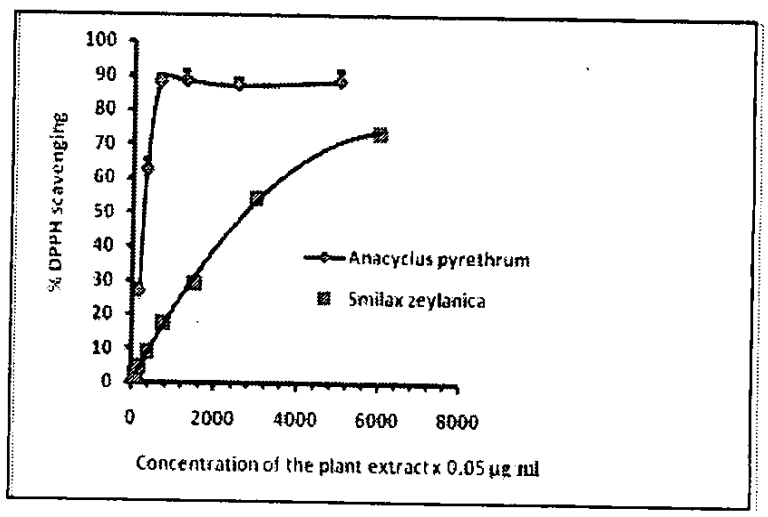
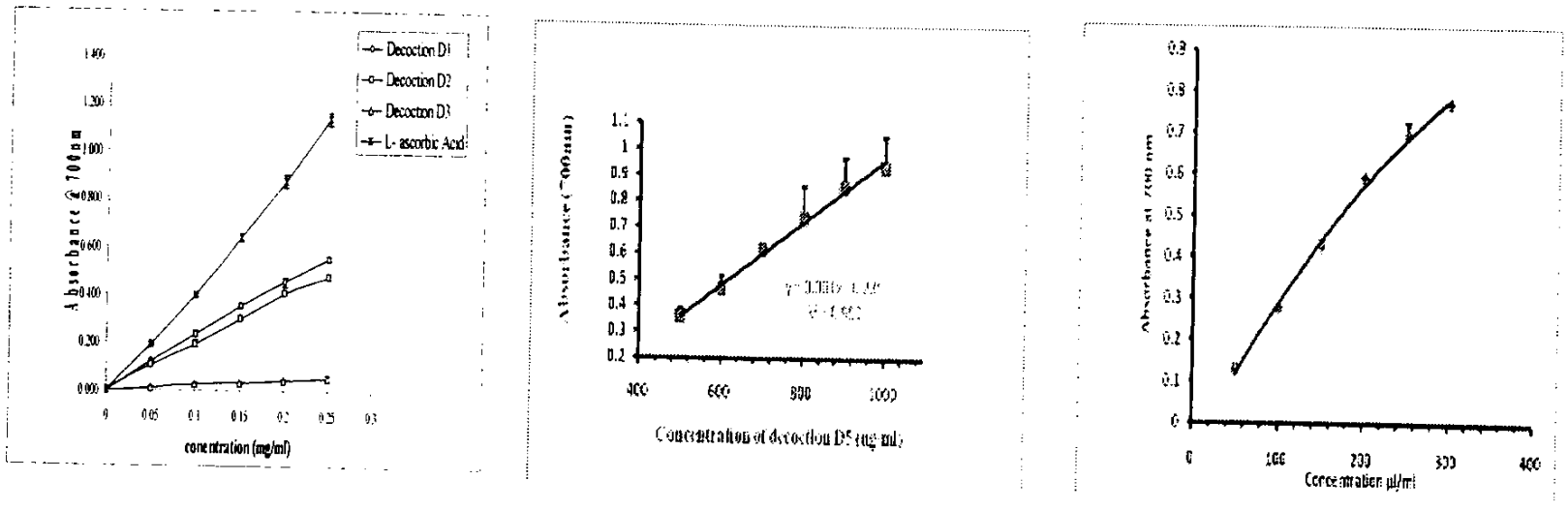


(f)

Figure 3.5.1 The dose response curve for % scavenging of DPPH of decoctions and plant extracts. a- D1 , D2 and D3; b-D4; c-D5; d-D6; e- *Smilax China, Bombax Ceiba, Anacyclus pyrethrum*, f- *Anacyclus pyrethrum* and rhizomes of *Smilax zeylanica*. **Final concentration of the plant extract was calculated considering the final volume obtained with the reagents added. For c, d, e and f original concentrationx0.05. Results are presented as mean ± SD.**



(a) (b) (c)

Fig.3.6.2 **Reducing power of decoctions D1, D2, D3 and ascorbic acid (a) n=6, and (b) D5 and ascorbic acid (n=3), and `Pranajeewa Oil'. Results are presented as mean ± SD**

Phenolic content and the EC50 for DPPH for the decoctions and plant extracts were exponentially correlated with R^2=0.8288.

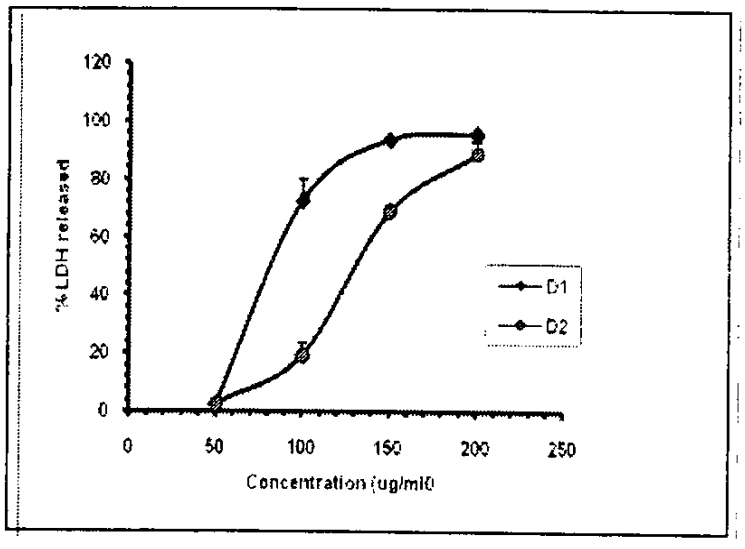
3.6.3 Cytotoxicity assays

3.6.3.1 Brine Shrimp assay

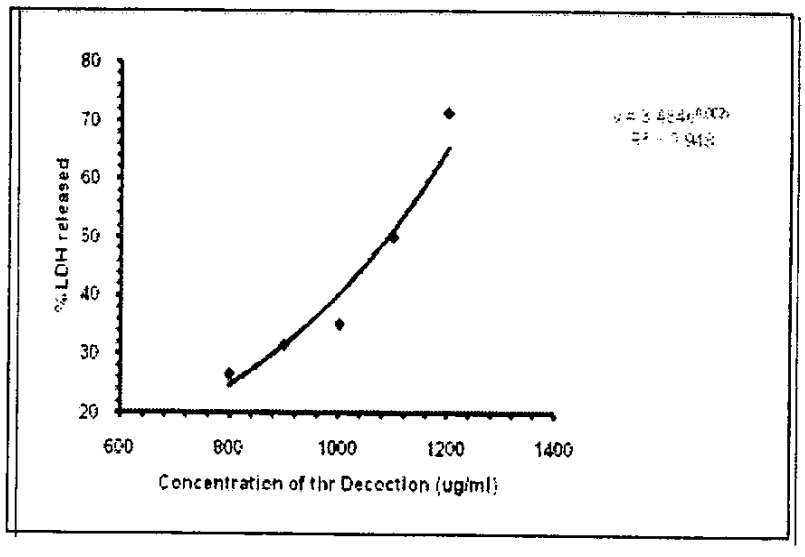
Brine shrimp assay is more convenient assay for drug toxicity and performed with D5, D6 and pranajeewa oil. Interestingly the cytotoxcity obtained for brine shrimp assay was relatively high compared with LDH and MTT assays for respective decoctions (Table 3.5.3).

3.6.3.2 LDH, MTT and protein synthesis assays

The cells showed cytotoxicty at varying degrees with all six decoctions and the EC 50 values for LDH and MTT are shown in Table 3.6.3 and illustrated in Fig. 3.6.3 and Fig 3.6.4. The highest activity was shown by the decoction 1. Sigmoid curves were observed for all decoctions investigated with increasing concentrations. The D6 showed a decrease in the total LDH over the concentrations of 600ug/ml producing a parabolic curve. Reproducible results were not obtained for pranajeewa oil for MTT or LDH. Protein content reflects the cell density and the percentage was calculated relative to the untreated cells as MTT assay. Cell lysate was evaluated for proteins in D4 and D6 by Lowry's method and D5 with SRB assay. The results are illustrated in Table 3.6.3.



(a)



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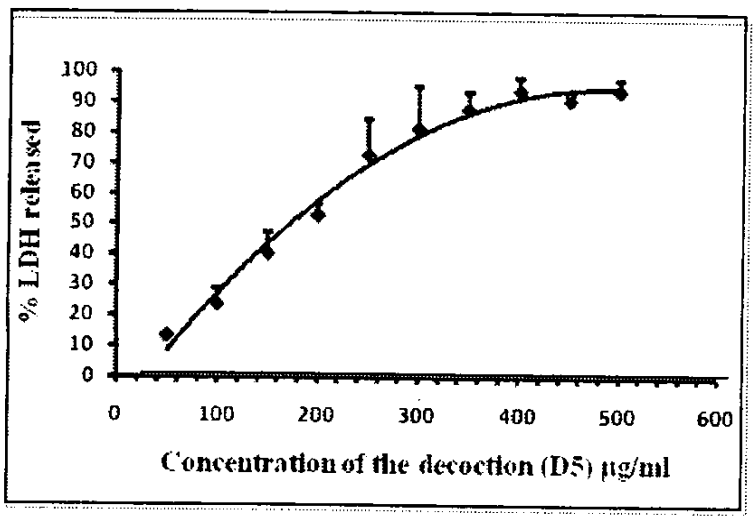
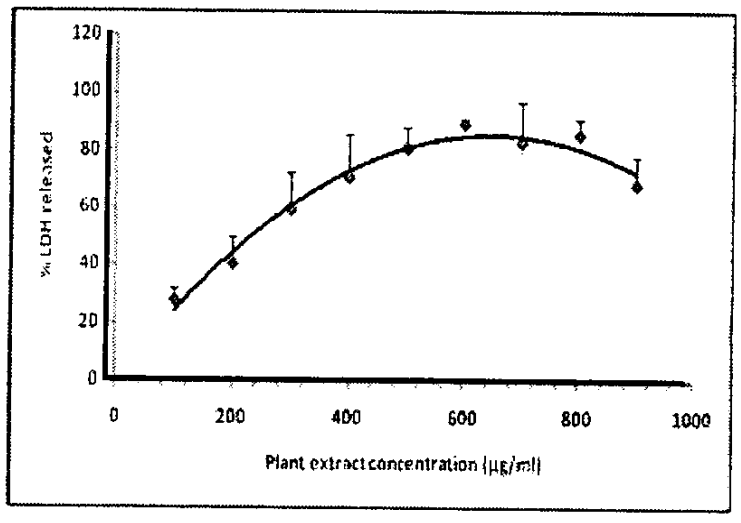
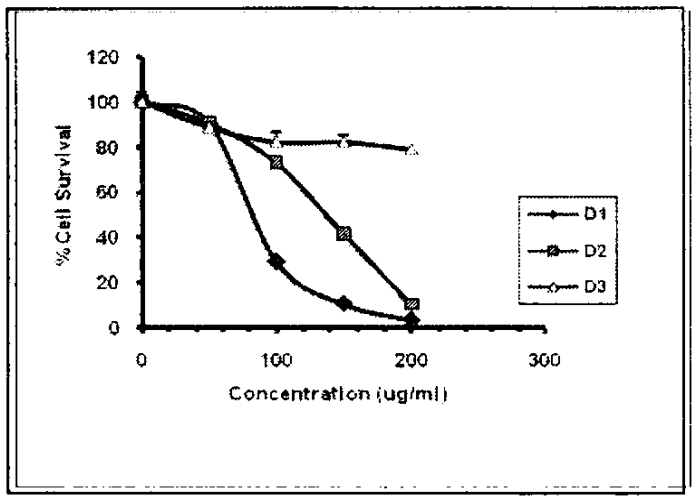


(c)

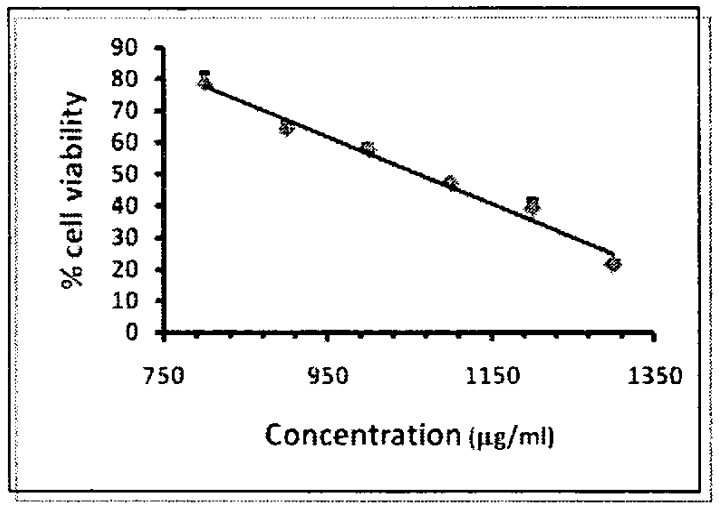


(d)

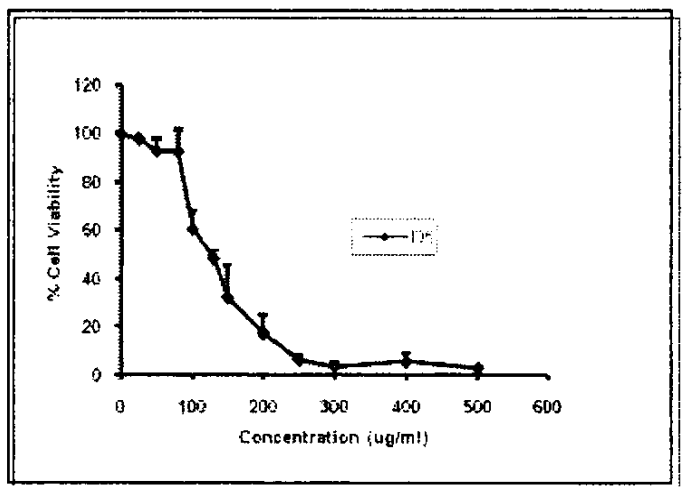
Figure 3.6.4: The percentage LDH released with the different concentrations of the decoction after the treatment of the *HEp*-2 or RD cell lines for a period of 24 hours with decoctions. The test was done in triplicates (A) and the linear part of the curve was used to calculate the EC_{50} values by linear regression analysis. a-D1 and D2; b- D4; c-D5 and d-D6



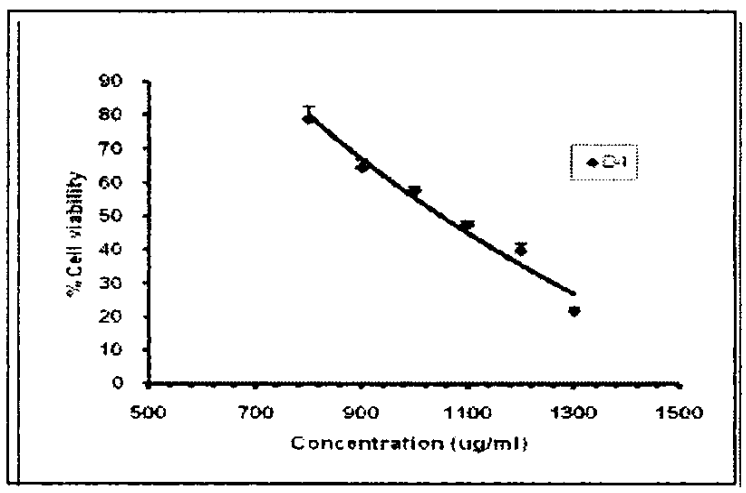
(a)



(b)



(c)



(d)

Figure 3.6.5: The effect of decoction at different concentrations on *HEp*-2 cell cytotoxicity as determined by the MTT assay. The experiments were carried out in replicates and the linear segment of the curve was used to calculate the EC_{50} values by linear regression analysis. a-D1 and D2; b- D4; c-D5 and d-D6

Table 3.6.3 EC50 values for LDH leakage and MTT assay in cell lines after exposure to decoctions D1, D2, D3, D4, D5 and D6 for 24 h. Data are presented as mean of the percentage LDH released to that of Total ±SEM (n=3).

	EC_{50} LDH (µg/ml)	EC_{50} MTT (µg/ml)	EC_{50} Protein Synthesis (µg/ml)	Brine Shrimp Assay (µg/ml)
Decoction D1*	94.4±4.02	80.58±2.58	NA	NA
Decoction D2*	129.7±3.4	132.0±1.16	NA	NA
Decoction D3*	>200	>200	NA	NA
Decoction D4**	1060±20	1230± 130	1540 ± 310 (Lowry's)	NA
Decoction D5**	178.88±16.84	140.69 ± 12.57	94.02 ± 1.90 (SRB)	2166.1±59.7
Decoction D6**	254.5±42.9	506.8±63.2	305.84±12.40 (Lowry's)	>500
Pranajeewa oil**	NA	NA	NA	13.0±0.8 (µL/ml)

*RD cells, ** *HEp* 2 cells. SRB Sulforhodamine B Assay. NA - not available

Phenolic content and the EC50 for LDH and MTT assays for the decoctions and plant extracts were exponentially correlated with R^2 >0.95.

3.6.3.3 DNA Fragmentation

DNA fragmentation was observed for D6 (Fig 3.6.6) for a single attempt at a concentration of 200 µg/ml. This method was not successful to the D1, D2, D3, D4 and D5 in the laboratory even to the positive control. This could be related with low concentration of DNA fragments which depend on number of apoptotic cells.

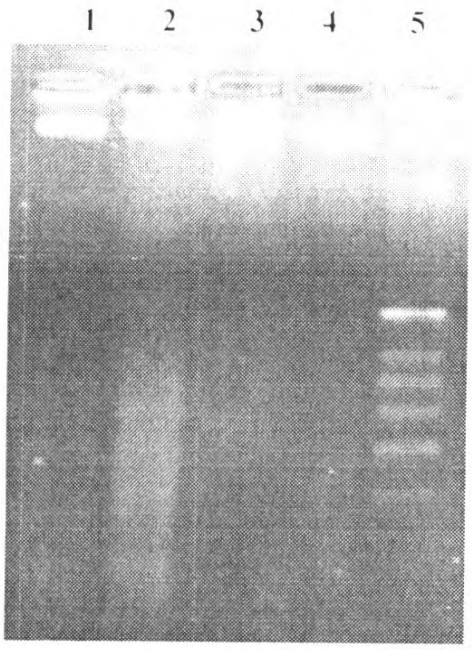


Figure 3.6.6 Agarose gel electrophoresis shows DNA fragmentation indicating induction of apoptosis by D 6 in *HEp 2* cells. Lane 1: negative control with untreated cells, lane 2: treated with 200 µg/ml, lane 2: treated with 450 µg/ml , Lane 4: Positive control with campothesin (5mM, 20µl), Lane 5: DNA molecular weight marker.

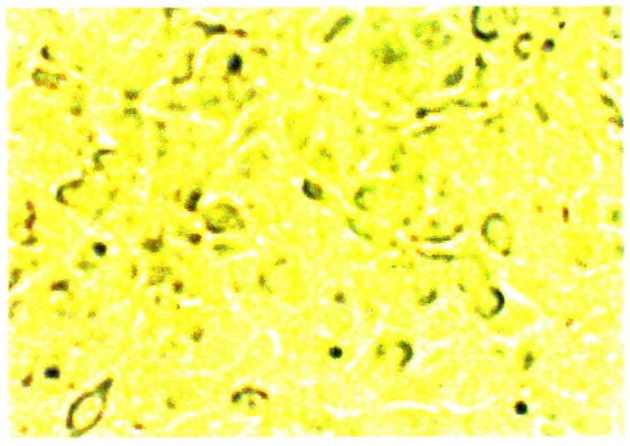
3.6.3.4 Light microscopic observation

After 24 h of treatment morphology of the treated cells were studied with all the decoction and compared with untreated cells. All the decoctions and pranajeewa oil induced morphological changes of the cancer cells. Microscopic observations reveled, rounding and detachment of cells which was more prominent with D1 and D2 at low concentrations compared to the other plant material investigated Fig 3.6.7 and 3.6.8. Progressive structural alterations and reduction in RD cell density with D1, D2 and *HEp 2* cell with D4 (Fig 3.6.10) D5 (Fig 3.6.11), D (Fig 3.6.12) and 'pranajeewa oil' (Fig 3.6.13) populations were observed with increasing concentrations to different degrees for all the decoctions studied.

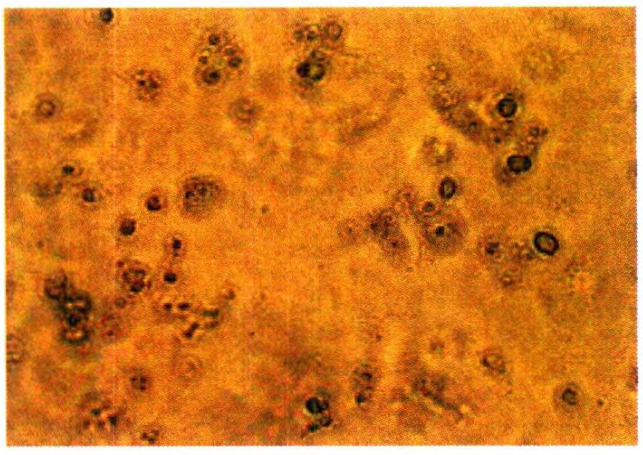
3.6.3.5 Fluorescence microscopy using Acridine orange/ethidium bromide (AO/EB) double staining.

Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA (Baskic', 2006). Ethidium/Acridine staining was performed with *HEp 2* cells after treatment with D5 and D6 for 24h.. The morphological changes were observed were shown in Figure 3.16.17 and 3.16.18, normal live cells were bright green in color whereas drug treated *HEp 2* cells were bright orange in color with condensed nuclei. Besides, normal nuclei showed chromatin with an organized structure, while apoptotic nuclei showed highly condensed chromatin in *HEp 2* cells treated with D5 and D6.

A

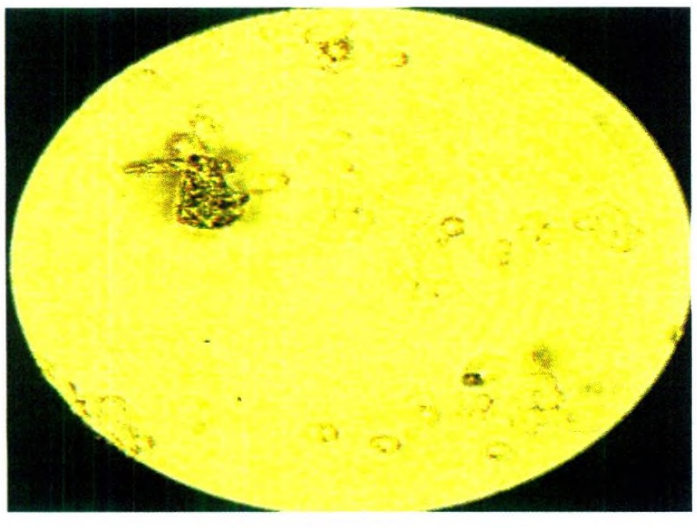


B



C

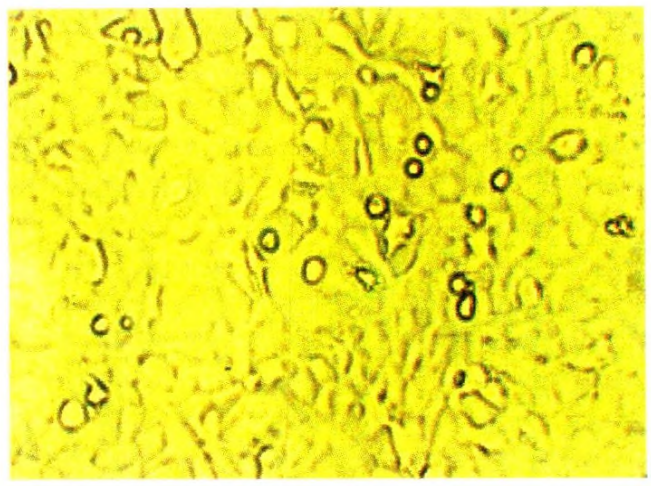
D



E

Figure 3.6.6 Images illustrating morphological changes in RD cells after treatment with different concentrations of decoction D1. (A) control (B), (C), (D) and (E) are D1 treated RD cells at concentrations 50, 100, 150 and 200 μg/ml respectively (200 X)

(A)



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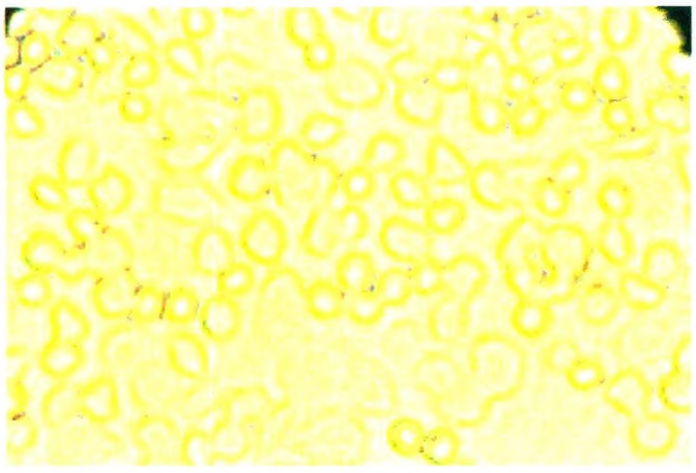
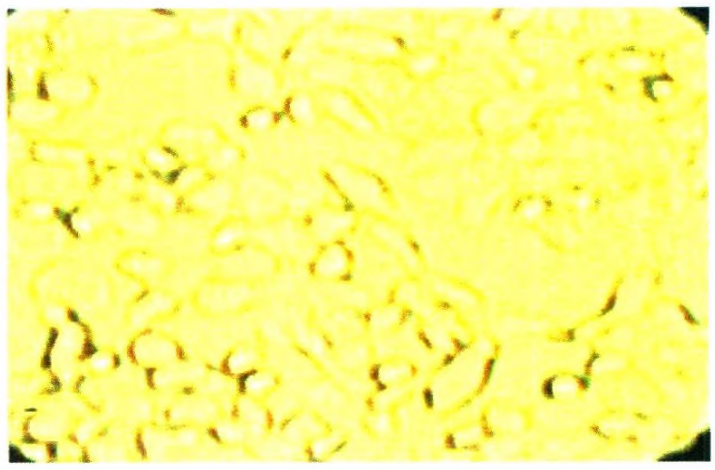


(C)



(D)

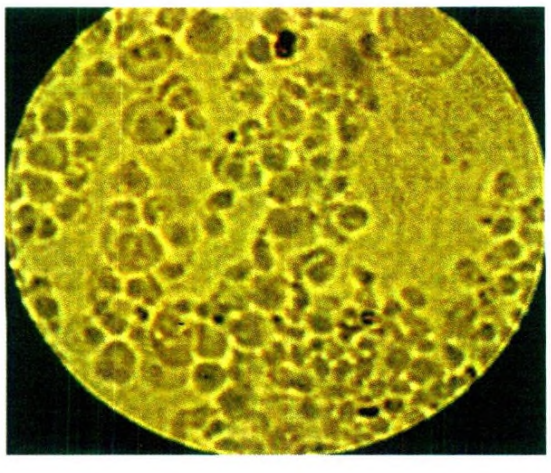
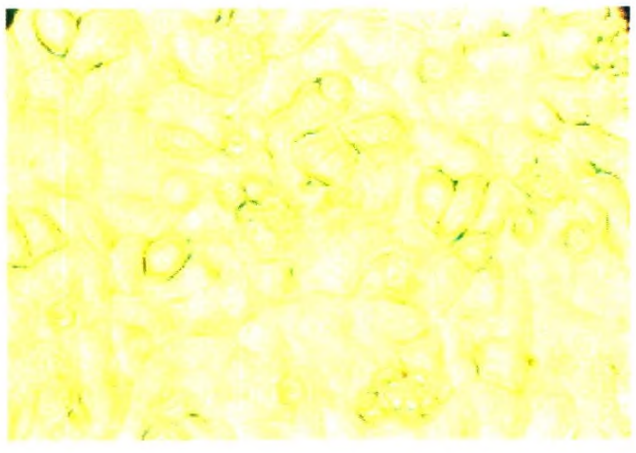


Figure 3.6.7 Images illustrating morphological changes in RD cells after treatment with various concentration of decoction D2. (A) Control (B), (C), (D) and (E) are D2 treated RD cells at concentrations 50,100,150 and 200 μg/ml respectively (200 X)



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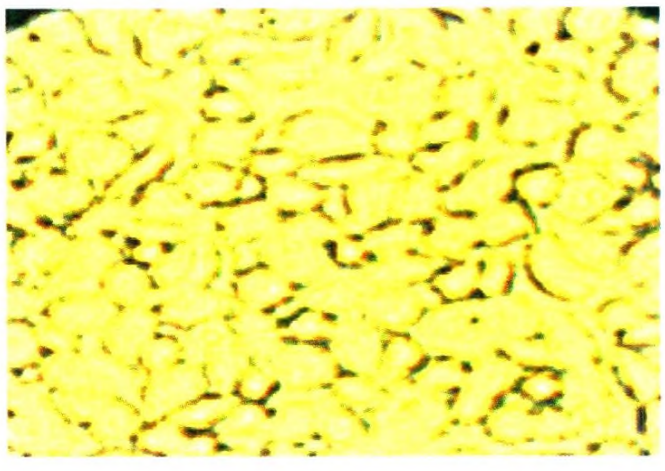

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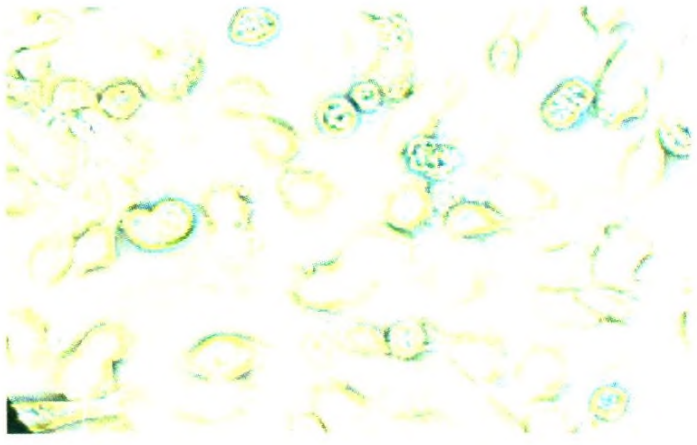
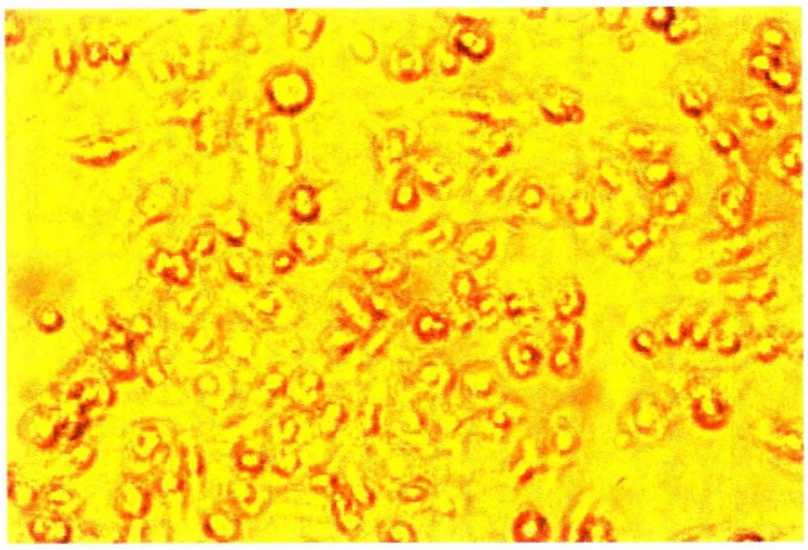


(C)

Figure 3.6.8 Images illustrating morphological changes in RD cells after treatment with decoction D3 and positive control camptothecin. (A), (B), and (C) are Control, D3 (200 μg/ml) and camptothecin (12.5 μM) treated cells respectively (200 X)



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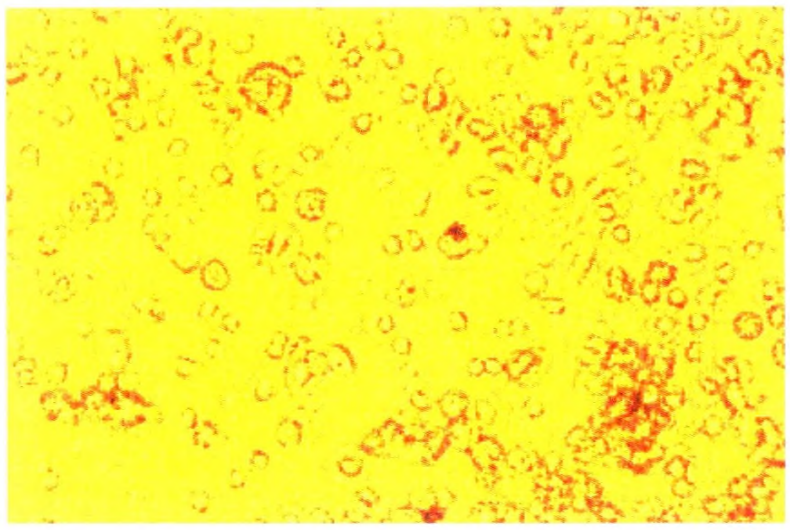
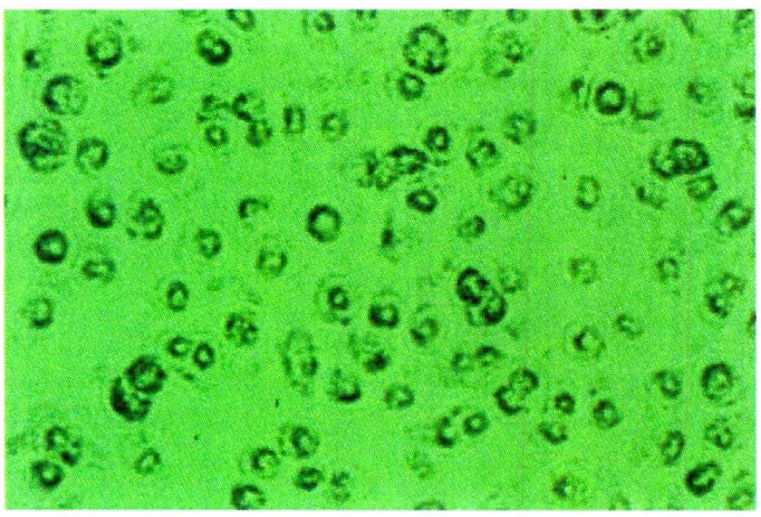
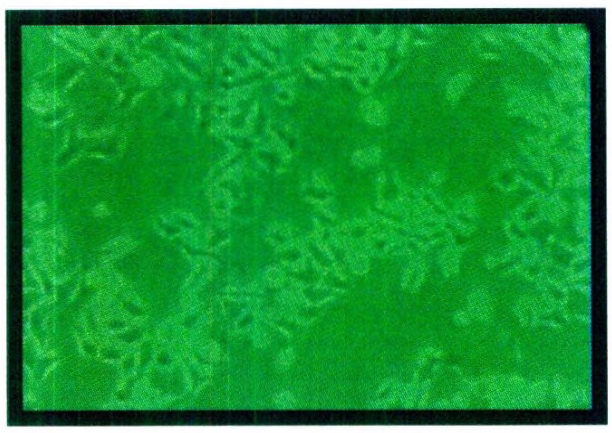


(b)

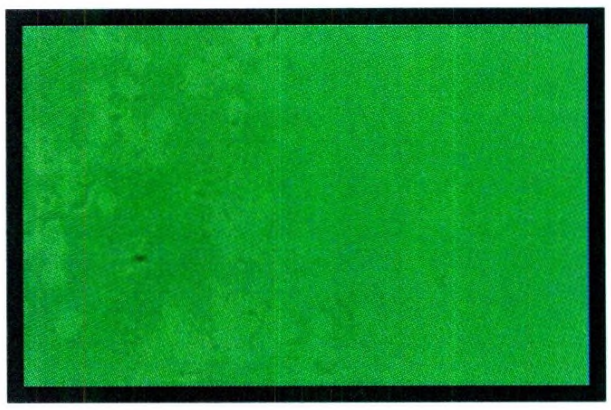


(c)

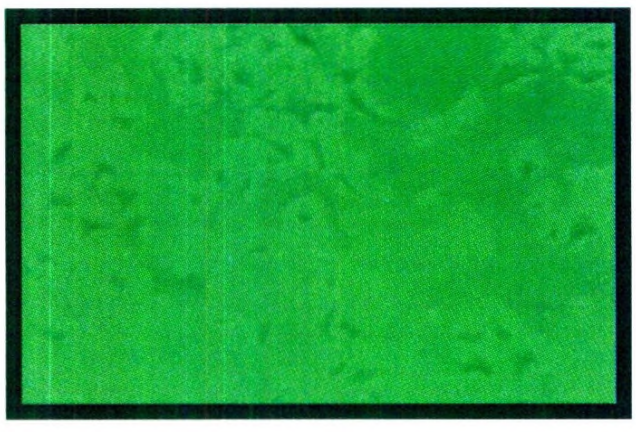
Figure 3.6.9: The morphological changes of Hep-2 cells after 24 hour treatment with Decoction 4 at concentrations of 800, 1000 and 1200 µg / ml.



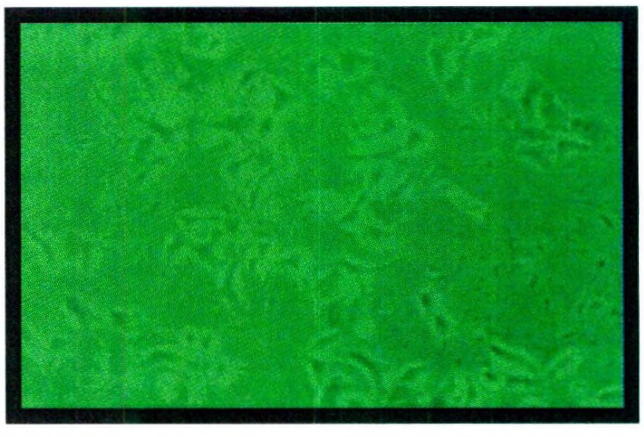
(A)



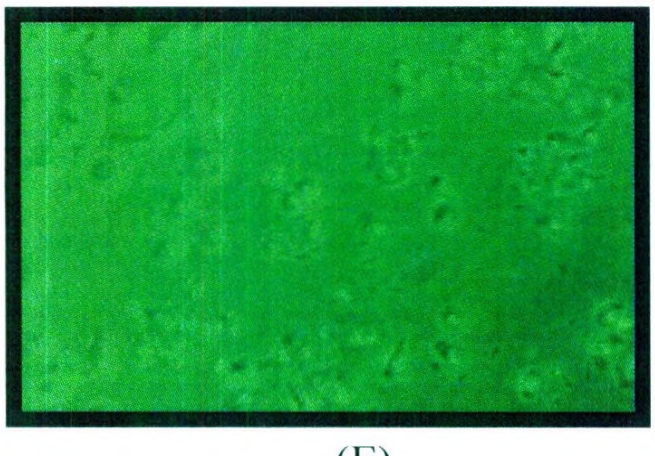
(B)



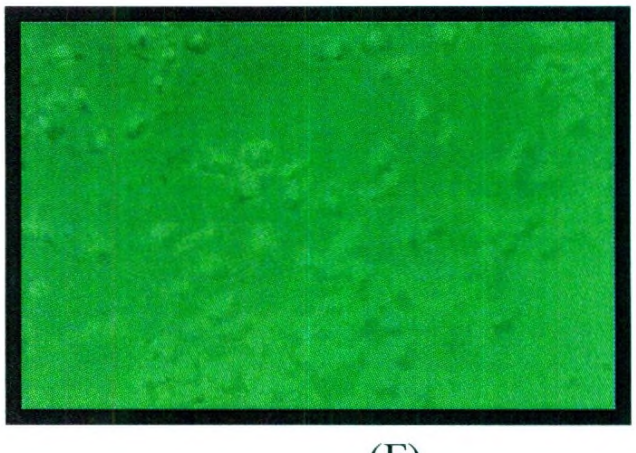
(C)



(D)



(E)



(F)

Figure 3.6.11: Light micrographs (Phase contrast) of cultured HEp-2 cell line 24 hours of incubation: (A) - Untreated control cells (B) – Cells incubated with camptothecin (5mM; 25µl) as positive control. (C), (D), (E) and (F) – cells incubated with 50, 100, 800 and 1000µg/ml of the decoction 5 (D5) respectively. (Magnification 100X)

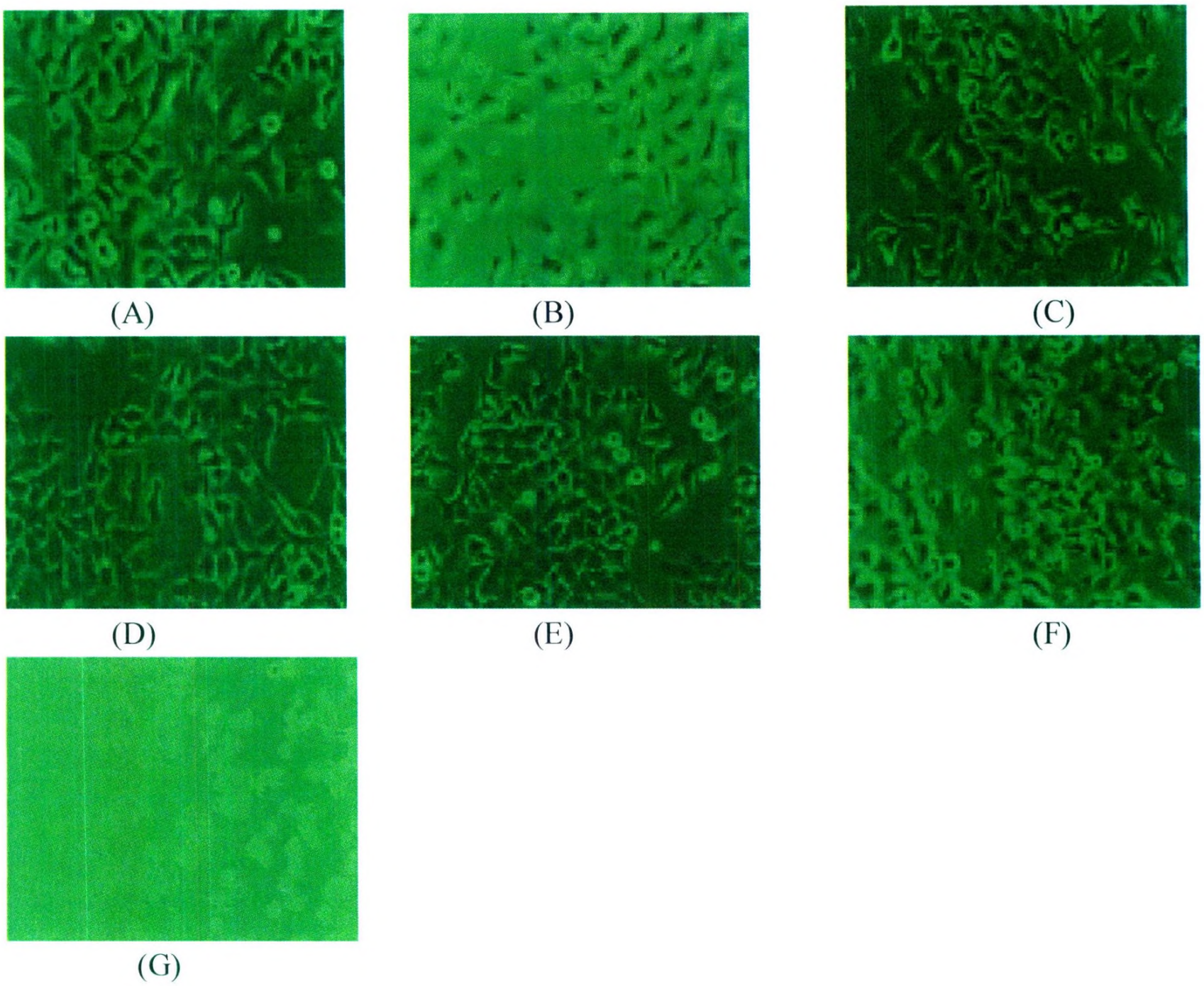


Figure 3.6.10: Light micrographs of HEp-2 cell line after 24 hours of incubation with the *Flueggea leucopyrus* extracts (D6) at different concentrations. (A) – Untreated cells: (B) – Cells incubated with Camptothecin (5mM; 25µl) as positive control. Cells treated with a drug concentration of 100µg/ml shows minimum cell death (c): Cells incubated with drug concentrations at 300, 400 and 500 µg/ml are illustrated in D, E and F respectively. Cell death was dose dependent and it increased with the increase in concentration. The highest concentration of drug (1000 µg/ml) displayed the greatest level of cell death as indicated in (G). (Magnification 100X)

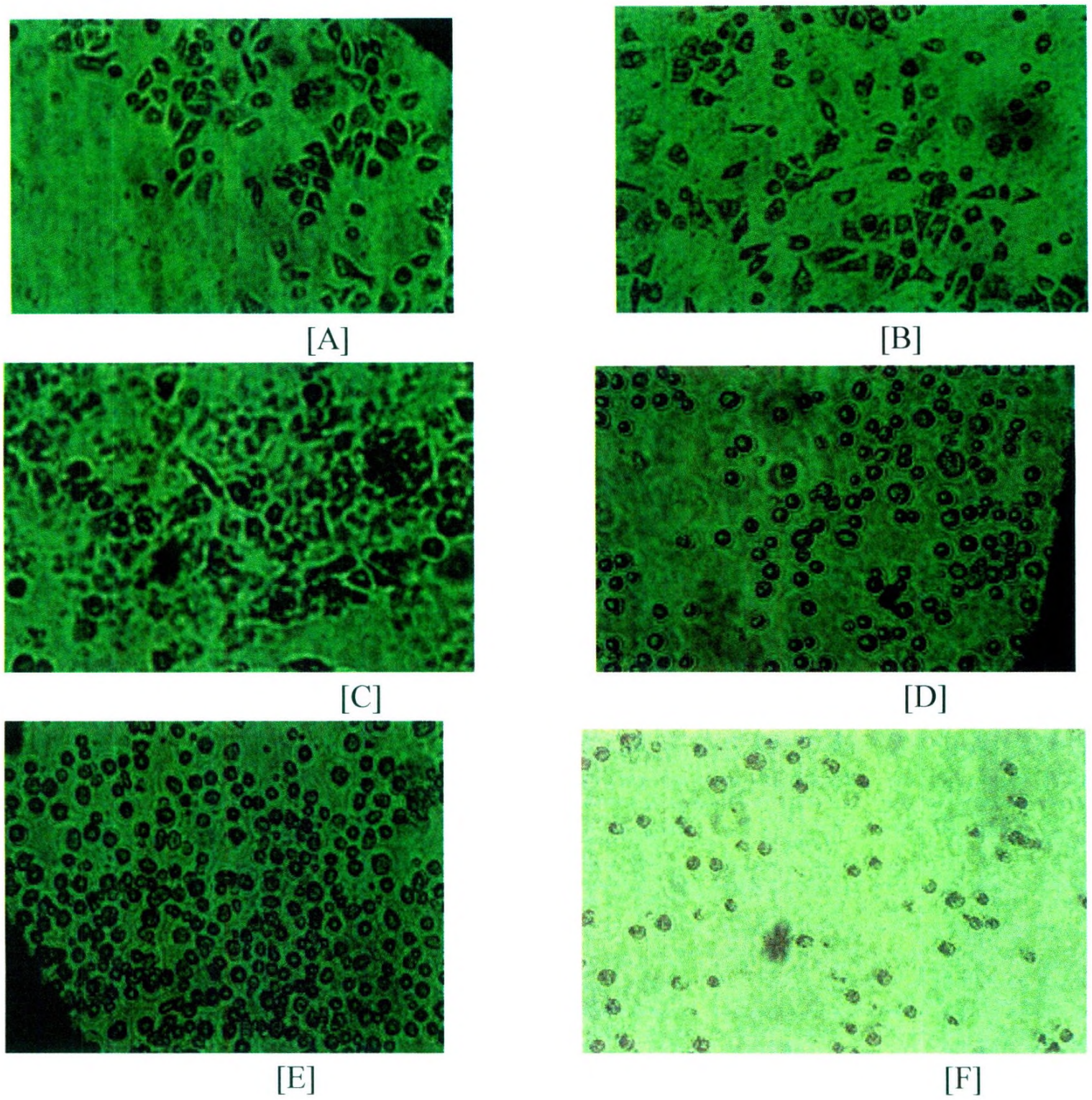
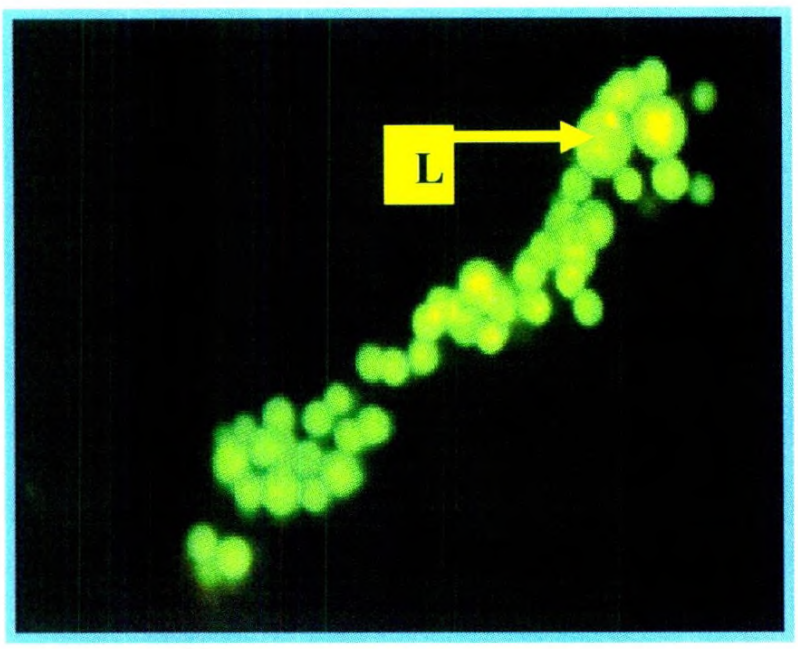
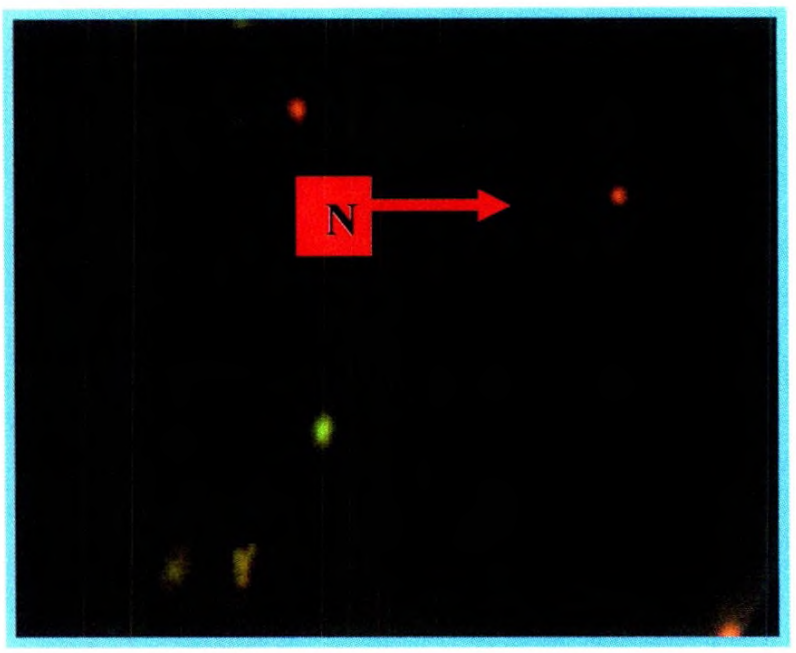


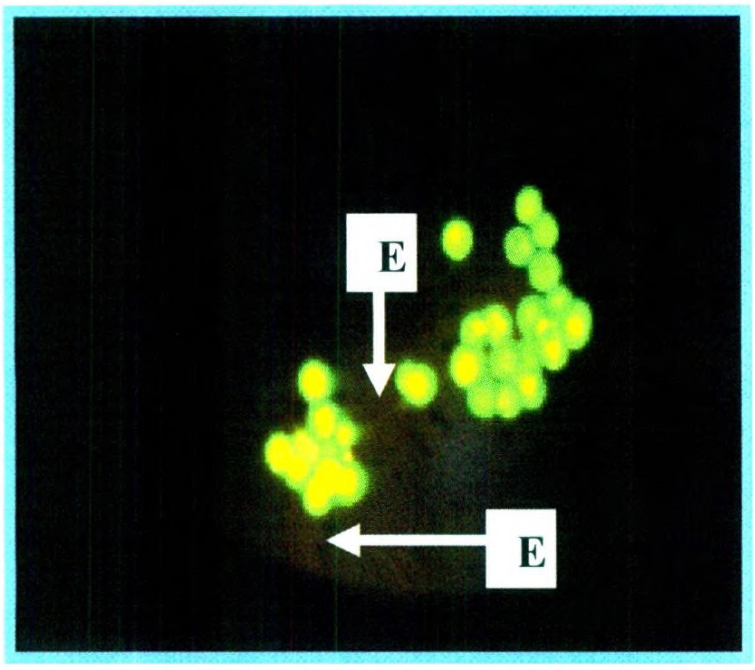
Figure 3.6.12: Images illustrating morphological changes of Hep2 cells after 24 hour treatment with Pranajeewa oil. A: control. B, C, D and E: oil concentration at 0.5, 2.5, 4.0 and 4.5µl/ml, F: positive control (camptothecin)



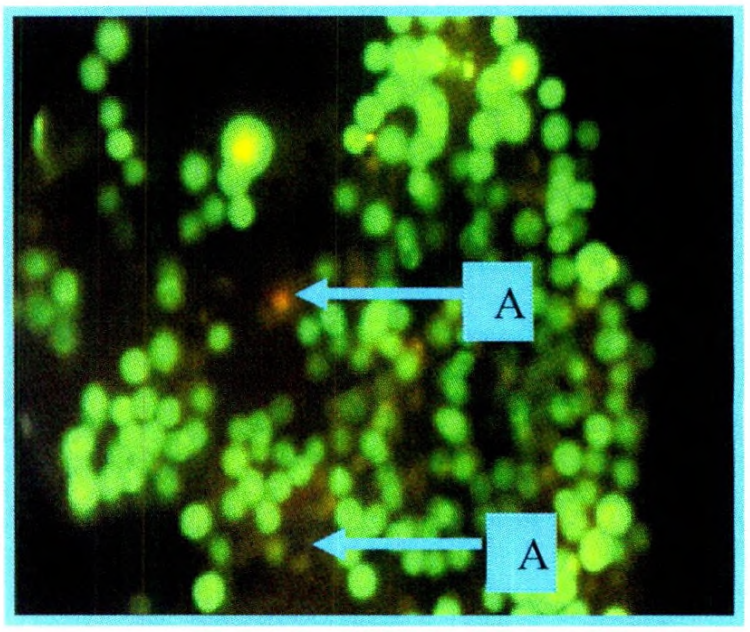
(A)



(B)

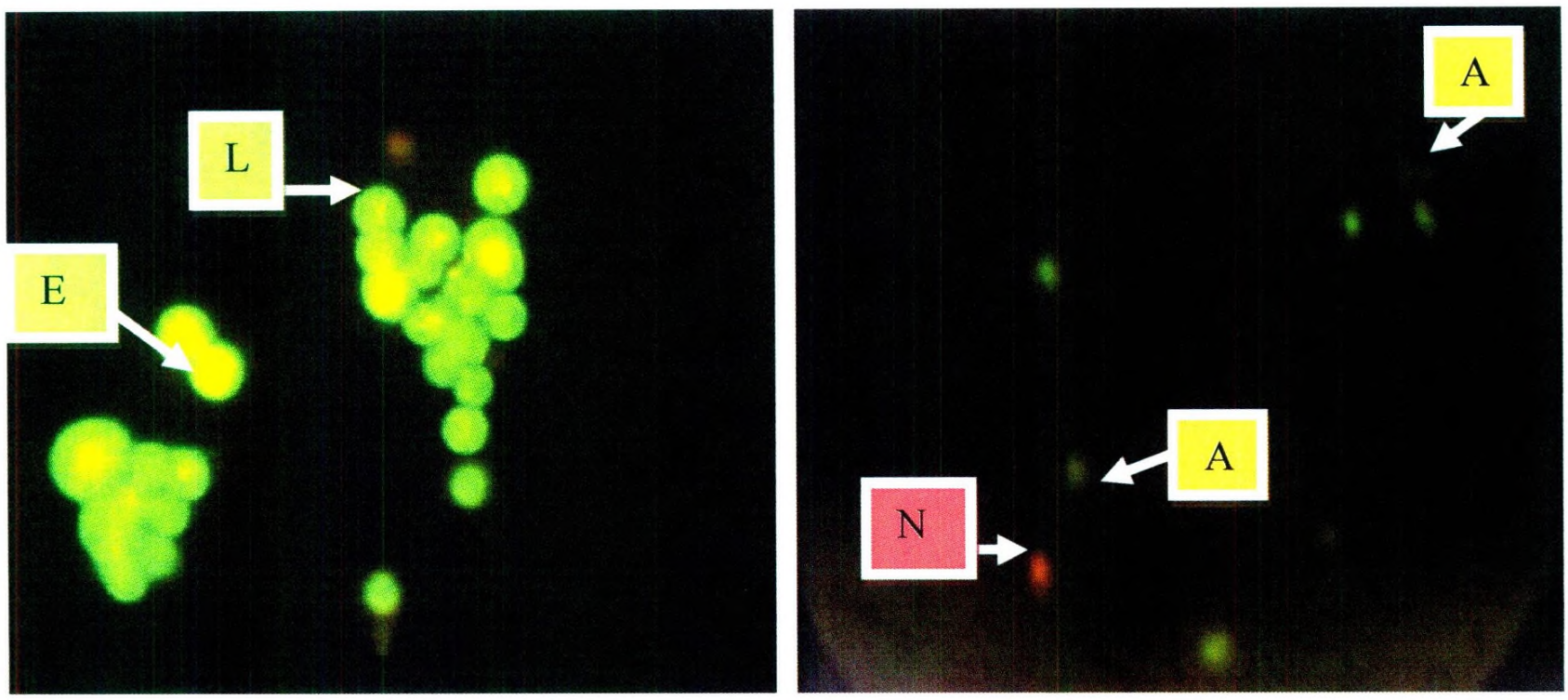


(D)



(C)

Figure 3.6.16: Images of cells using acridine orange-ethidium bromide staining. (A) - Untreated cells and (B) - Cells incubated with camptothecin (5mM; 25µl) as positive control. The effect induced by the decoction 5 (D5) of different concentrations. (A) & (B) - Cells incubated with 50µg/ml and 150µg/ml respectively. Yellow arrows next to "L" point to viable cells; red arrows next to "N" indicate necrotic cells; white arrows next to "E" indicate early apoptotic cells; and blue arrows next to "A" indicate late apoptotic cells. (Magnification 100X)



(A) (B)

Figure 3.16.17: Identification of apoptotic cells using acridine orange-ethidium bromide staining and the effect induced by *Flueggea leucopyrus* extract of different concentrations. (A)- Cells incubated with 200µg/ml and (B) - Cells incubated with 800µg/ml. "L" refers to live cells; "E" indicates early apoptotic cells; "N" points to necrotic cells and "A" indicates late apoptotic cells.

3.7 Discussion

We have a long history in use of plants as medicines in treatment of many diseases, which are recorded in Ola leaf inscriptions or inheritance from native and traditional doctors. Most of these medicines are of combination of many herbal materials or of a single plant extract. A combination of multiple chemopreventive agents or agents with multiple targets is considered to be more effective than a single agent (Parmar *et al* , 2010). A wide variety of plant metabolites including phenolic substances posses potent antimutagenic and anticarcinogenic activities (Surh, 1999). Selected prescriptions used by the traditional/native doctors for cancer treatment were investigated for phenolic content, antioxidant activity, and cytotoxicty since secondary plant metabolites are associated in prevention and treatment of many diseases.

The phenolic content showed a marked variation with a highest content associated with D1 and a decreasing order of D2=D5>D6>D4>D3. Individual analysis of Imbul Malliyam (*Bombax ceiba)* and Akkarapatta (*Anacyclus pyrethrum)* which are components of D4 showed higher levels of phenolic content (>30%). Gallic acid (GA) a plant phenolic acid, which has shown cytotoxic activites against cancer cell lines (Inoue *et al.*, 1995) was found at different concentrations (0.58-6.97mg/g) in all the decoctions analysed for gallic acid. In addition Cheena ala (*Smilax china*) and Imbul maliyam (*Bombax ceiba)* also showed the presence of GA among the four individual plant materials studied and the values were beyond the detection limits in Iramus (*Hemidesmus indicus*) and Akkarapatta (*Anacyclus pyrethrum*). Faried *et al* (2006) demonstrated that GA showed a significant inhibition of cell proliferation in a series of cancer cell lines with EC 50 values in the range of 0.01-0.80 mg/ml. The decoction 5, which composed of both *Thespesia populnea. L* and *Adenanthera pavonina.L*, showed a high flavonoid content of 42.40 ± 0.39 w/w % of EGCG equivalents. Ethanolic and aqueous extract of *Thespesia populnea* bark as reported by Parthasarathy *et al.*, (2010), contained, tannins, phenol and flavonoids., and the methonolic extracts of flowers contained flavanoid and phenol content of 21.05 (quercetin equivalents) and 31.1 (GAE) mg/g respectively (Saravanakumar *et al.*,2009).

Antioxidants play an important role in cancer prevention (Hou, 2003) hence phenolic compounds is postulated as effective antioxidant and anticancer agents (Surh, 1999). The DPPH scavenging ability is in order of D1>D2=D5>D6> D4 and the EC50 values were very high with D1, D2 and D5 and comparable to the ascorbic acid (6.4 ± 0.1 µg/ml). A previous study on *Smilax china* has shown that EC_{50} values of DPPH radical scavenging activity of its solvent extraction fractions of ethyl acetate, butanol and water, were 4.6, 8.7 and 9.6 µg/ml, respectively (Lee *et. al*: 2001) in contrast to the value (35.67 µg/ml) obtained for the present study. The water extract of *Bombax ceiba* (Gum) and *Anacyclus pyrethrum* in the present research study showed EC_{50} values of 15.47 and 15.01 µg/ml respectively; reflecting their contributions of DPPH scavenging ability to the Decoction 4. The phenolic content was correlated [$y = 1896.3x^{-1.5149}$ (r^2= 0.7719)] with the EC_{50} for DPPH radical scavenging activity, which suggests that the phenolic content of the decoctions and plant extracts has exponetially contributed for the scavnging of free radical in vitro. The EC 50 value for DPPH of pranajeewa oil was obtained at 79.84 ± 5.35 µl/ml. The DPPH radical scavenging

activity was as high as 81.06±1.43 % at 150 µl/ml concentration of the oil. The leaf of aqueous extract, flower and the ethanol extracts of stem bark of Neem which its oil is used in preparation of 'Pranajeewa' oil, exhibited EC_{50} for DPPH scavenging activity at concentrations 26.5, 27.9 and 30.6 µg/ml, respectively (Sithisarn *et. al*, 2005). Variations in the extent of antioxidant activities were observed for decoctions or plant extracts, between assay metods. The EC_{50} values obtained were 53.21 and 23.77 µg/ml for ribose method and for nitric oxide scavenging generated from SNP were 4.82 and 14.42 µg/ml for decoction 5 and *Fleuggea leucopyrus* (D6) respectively. This shows that, though the higher inhibition showed for NO scavenging activity by leaf extract of *Fleuggea leucopyrus* compared to the decoction 5, DPPH and OH radical scavenging activities were higher with D5. Similar to their antioxidant activities, the reducing power of each decoction increased with increasing dosage but showed lower reducing ability compared with L Ascorbic acid. The decoction D3 showed very low reducing power as well as low antioxidant activity, which can be associated with its low phenol content.

The cytotoxic potential of the six decoction currently used in the treatment of cancer was studied by release of LDH, MTT assay, protein synthesis and characteristic morphological changes observed in microscopy. The LDH activity is given as a percentage of LDH found in the culture medium with respect to the total LDH (Lopez *et al*, 2003). The MTT assay measures the cell proliferation rate. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells (Mosmann,1983) by mitochondrial dehydrogenase and directly proportional to the number of surviving cells. Parabolic curve obtained for % LDH release of *Fleuggea leucopyrus Wild* (D6) suggest that, the cell death may have occurred within a short period of time after the extract exposure and therefore no LDH release due to the dead cells. The values for EC 50 for LDH and MTT for the decoctions were well correlated exponentially with their phenolic content, reflecting the contribution of phenolic content to the cytotoxiciy. It can also suggest that gallic acid which is a phenolic substance present in all the decoctions studied, may have contributed synegestically to the cytotoxicity of cancer cell lines. It has been reported that gallic acid has shown cytotoxic activites against cancer cell lines (Inoue *et al*., 1995) and Kaur and coworkers have identified gallic acid present in Triphala plays an important role in inducing cytotoxicity and apoptosis in cancer cell lines. Samudio *et al*. (2005) 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 which is an ingrdient in D1 and D2. *In vitro* screening on *Toddalia asiatica Lam* (Iwasaki, 2006), ricin found in *Ricinus Communis L*. seeds (Patočka and Středa; 2003) ethanolic extract of *Boerhaavia diffusa* (Mehrotra *et.al*.,2002) have shown cytotoxicty in different animal cell lines, supporting the contribution of the constituent for cytotoxicity of D4. MTT or LDH with pranajeewa oil did not show reproducibility of the experiments. The formation of droplets of oil with aqueous medium and aggregation of oil on the top of the medium and along the wall of the well does not provide a fixed concentration to the cells resulting poor reproducibility.

The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity, (Meyer *et al*, 1982) since bioactive compounds are often lethal to brine shrimps. The EC 50 values obtained for Brine shrimp assay is comparatively high compared to invitro cytotoxicty assays determined by LDH, MTT and protein synthesis for respective decoctions. Though the cytotoxicity experiments with cells were not successful , brine shrimp assay worked well with pranajeewa oil with excellent reproducibility.

The morphological characterization of the treated cells shows that the mode of action of cell death induced by decoctions was mediated through apoptosis. Enlargement of these pictures exhibited the apoptotic features of cytoplasmic vacoulation and condensation, and the presence of apoptotic bodies. These observations explain the apoptosis-inducing effect of decoctions. The total phenolic content and DPPH radical scavenging activity correlate the cytotoxicity which provide explanations to this morphological findings. To investigate further the type of cell death induced by the decoctions, the cells were stained with AO/EB for D5 and D6 since the method applied at the latter stage of the research. AO/EB staining allows the identification of viable, apoptotic and necrotic cells based on color and appearance. The florescent tagged dye diminished within few second after staining and method was successful with D5 and D6. Photographic observation of cell morphology of this study consistent with the cytotoxic determinations of MTT assay, LDH activity and protein synthesis. Further D6 showed the characteristic DNA fragmentation in apoptosis.

3.8 Conclusion

All the six decoctions showed cytotoxicity at different levels of concentrations. Among them D1 , D2 and D5 showed higher antioxidant activity compared to the other decoctions studied. All the decoctions are very effective in inducing cell death on cancer cell lines except D3 and D4. Exponential relationship shown by polyphenols suggests that polyphenols play an important role in free radical scavenging activity and cytotoxicty. The parallel use of techniques in determining cytotoxicty, allows us to observe the structural changes occur in cell death induced by decoctions is via apoptosis.

3.9 Antioxidant activity and Cytotoxicity of *P. cystidiosus* mushroom extracts/ fractions

Summary

This study was carried out to evaluate the antioxidant activity and cytotoxicity against *HEp 2* cancer cell line of Pleurotus cystidiosus. Fresh *P. cystidiosus* mushroom was extracted by acetone (A), dichloromethane and hexane. *HEp 2* Extraction and fractionation of *P. cystidiosus* mushroom was carried out using the procedure described by Vasudewa *et al.* (2008). Briefly, fresh *P. cystidiosus* mushroom was extracted into acetone, dichloromethane and hexane. Acetone extract was labeled as "A". Extract A was extracted into hexane, dichloromethane, ethyl acetate and the remaining water fraction was labeled as "A4". Fraction A4 was further fractionated into three fractions, A4-1, A4-2 and A4-3 using a reverse phase column. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) radical scavenging activity and nitric oxide radical scavenging activity were investigated to evaluate its health beneficial effects. The highest scavenging capacities for *P. cystidiosus* mushroom extracts fractions for both experiments were shown by A4-2 and A4-3. The values obtained for EC50 for DPPH scavenging activity of A4-2 and A4-3 were 0.81 and 0.82 mg/ml, respectively, and for niric oxide scavenging activity, EC50 was 0.87 & 0.61 mg/ml for A4-2 and A4-3 respectively. Cytotoxicity studies were carried out for all fractions over a concentration range of 0.5-5.0 mg/ml after 24 hour exposure to *HEp 2* cells. The results on cytotoxic effects based on MTT and LDH assays have shown that the same two extracts, A4-2 and A4-3 to have the highest activity. The percentage LDH released by the extracts/fractions of *P. cystidiosus* after exposure to *HEp 2* cell line at a concentration of 5 mg/ml were 32.7±3.0, 32.2±2.5, 8.3±0.2, 51.5±1.5 and 48.7±2.9% for A, A4, A4-1, A4-2 and A4-3 respectively. The EC50 was obtained only for A4-2 and A4-3 within the concentration range studied and the values were 3.3 and 3.9 mg/ml respectively. The nitrite levels of the cell supernatant treated with mushroom extract/fractions were studied and the fractions A4-2 and A4-3 resulted in dose-dependent decrease compared to negative control. A dose dependant decrease in protein synthesis was observed for A4, A4-2 and A4-3 fractions. However 50% inhibition (EC 50) was observed only for the fraction A4-2 and A4-3 with values of 3.3 and 1.3 mg/ml respectively. These results were reinforced by the cell morphological changes observed using an inverted fluorescence microscope of the treated cells.

3.10 References

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3.11 Problems if any, encountered during the implementation of the project

- Receiving the chemicals sometimes delayed
- Obtaining of prescriptions of decoctions from traditional doctors which are effective in treatment in cancer therapy was a difficult task.
- The study was sometimes got delayed (over a month or more to culture new batch of cells) due to the contamination of cells. The power failure occurred during the week ends is one reason for the contamination and unavoidable.
- Since there is no camera unit for the microscope, the photography service was strictly depended on the availability of the photographers.
- The techniques in determination of cytotoxicty were new to the laboratory and trial and error processes consuming time (for cell growth).

Section 4

Impact of Research results:

i) Relevance of results achieved to scientific advancement

This work included of screening of antioxidant activities and cytotoxicty of selected decoctions currently used in cancer therapy by traditional doctors. Antioxidant activity was well correlated with the cytotoxicity and can be used as indicators in cytotoxicty of a plant extract before initiating the study with cancer cell lines since animal cell culture studies are expensive.

Many current techniques regarding apoptosis were developed in the laboratory and many postgraduate students were trained in the area of animal cell culture and evaluation of cytotoxicity.

Capacity Building: Seven Postgraduate students and one undergraduate student were developed the techniques in animal cell culture and cytotoxicyty work.

ii) Relevance of results achieved to national/socio-economic development

Results of this project will be benefitted to the development of our traditional medicine used for cancer therapy in Sri Lanka. The results will be very useful for clinical trials to select effective decoctions in cancer therapy.

The results of D5 was presented in Bandaranayake Memorial Aurvedic Institute, Nawinna.

iii) Dissemination/application of research output

One full paper was published. In addition the work was presented in national and international forums (Section 5).

Section 5

Miscellaneous

i) List of major equipment acquired during the project period and their functionality

None

ii) List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)

Publications/Communications arising from the project during the reporting period

1. Inoka P. Menikpurage, S.S.S.B.D.P. Soysa, D.T.U. Abeytunga The Journal is Pharmaceutical Biology (submitted)

2. M.G.A.N.Perera, S.S.SB.D.P Soysa, D.T.U Abeytunga, R Ramesh (2008). Pharmacognosy Magazine Vol 4, 172-181, Antioxidant cytotoxic properties of three traditional decoctions used for the treatment of cancer in Sri Lanka.

Communications with published abstracts

1. L.I.K Silva and S.S.S.B.D.P. Soysa. (2010). "Antiproliferative activity and induction of apoptosis by a decoction prepared from *Adenanthera pavonina and Thespesia populnea*" International Conference on Stem Cells and Cancer (ICSCC-2010): Proliferation, Differentiation and Apoptosis", 11-14 December, Pune. India.(Accepted)

2. L.I.K Silva and S.S.S.B.D.P. Soysa . (2010). Antioxidant and cytotoxic activities of a decoction prepared from *Adenanthera pavonina* (madatiya) *and Thespesia populnea* (gansuriya). Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science. Accepted for presentation

3. A.A.S.Adikary and S.S.S.B.D.P. Soysa, (2010). Comparison of cell viability of black, green and white tea (*Camellia sinensis*) manufactured in Sri Lanka. Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science 2010 (Submitted)

4. Adikary A.A.S and Soysa S.S.S.B.D.P. (2009). High grown Sri Lankan black tea extract without the polyphenolic components does not show cytotoxic and antioxidant activities. Fourth Global Summit on Medicinal Aromatic Plants: Sarawak. Kuching Malaysia.1st-5th December

5. Adikary A.A.S and Soysa S.S.S.B.D.P (2009) Comparison of the antioxidant activity of Black, Green and White Tea Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science

6. Weerasekekara, S.M, Soysa S.S.S.B.D.P (2009). Study on the Antioxidant activity of a Traditional Herbal Oil used for Cancer therapy in Sri Lanka. Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science

7. Ihalagamage, P.J and Soysa S.S.S.B.D.P. (2008). 1st International Conference in Holistic Medicine-Kottayam, Kerala, India. August 21-23.: Study on antiproliferative activity of a selected decoction used for cancer therapy in Sri Lanka.

8. B.M.MJ.Mendis, M.D.J Wijebandara and S.S.S.B.D.P Soysa **(2008).** Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science. Characterization of Biological Activity of *Fleuggea leucopyrus* Wild (Katupila)

9. Manikpurage, I.P, Abeythunga D.T.U and S.S.S.B.D.P Soysa (2008). Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science. Anti-oxidant activity of *Pleurotus cystidiosus*

10. De Silva Savan Kalna, Ariyadasa Hirantha and Soysa S.S.S.B.D.P (2008). Proceedings of the Annual Sessions of Sri Lanka Association for Advancement of Science. Study on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity in relation to the phenolic and gallic acid content in four medicinal plants used for cancer therapy in Sri Lanka

11. M.G.A.N. Perera, S.S.S.S.B.D P. Soysa, A.A.S. Adikary, D.T.U. Abeytunga (2007) Total phenolic content and Gallic acid content strongly associate with antioxidant and cytotoxic properties of three traditional decoctions used for cancer Society for Integrative Oncology. Fourth International Conference Expanding Horizons in Collaborative Cancer Care, San Francisco, California, November 15-17, 2007.

Section 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and Subsistence and Miscellaneous)

Breakdown of Funds provided

Date	Cheque Number	Total	TA	Consumables	Travel/Sub -	Miscellaneous	Equipment
28.11.05	562601	231,500.00	18,000.00	-	1,000.00	7,500.00	205,000.00
09.01.06	568954	495,000.00	-	495,000.00	-	-	-
07.02.06	653008	495,000.00	-	495,000.00	-	-	-
14.02.06	656525	26,500.00	18,000.00	-	1,000.00	7,500.00	-
03.04.06	660831	6,000.00	6,000.00	-	-	-	-
05.07.06	568922	236,500.00	18,000.00	210,000.00	1,000.00	7,500.00	-
14.09.06	669053	36,750.00	24,000.00	-	2,250.00	10,500.00	-
29.05.06	527254	236,000.00	24,000.00	200,000.00	1,500.00	10,500.00	-
05.12.06	540429	33,000.00	24,000.00	-	1,500.00	7,500.00	-
19.03.07	571203	33,000.00	24,000.00	-	1,500.00	7,500.00	-
29.06.07	586428	33,000.00	24,000.00	-	1,500.00	7,500.00	-
	Total	1,862,250.00	180,000.00	1,400,000.00	11,250.00	66,000.00	205,000.00

Breakdown of Funds utilized

	Received	Spent Total	Balance
TA	180,000.00	120,000.00	60,000.00
Consumables	1,400,000.00	1,491,489.75	(91,489.75)
Missellaneous	66,000.00	71,838.00	(5,838.00)
Equipment	205,000.00	62,665.00	142,335.00
Transport	11,250.00	-	11,250.00
Total	1,862,250.00	1,745,992.75	116,257.25

Yearly Breakdown of Funds used

F/Year	2006	2007	2008	2009	2006-2009
Chemicals	75,559.00	521,627.00	281,919.25	33,129.60	912,234.85
Equipment	13,945.00	-	-	48,720.00	62,665.00
Consumables	57,500.00	475,179.90	46,575.00	-	579,254.90
Miscellaneous	14,979.50	9,954.50	8,152.00	26,353.00	59,439.00
Services	-	11,399.00	-	1,000.00	12,399.00
Technical Officer	-	-	120,000.00	-	120,000.00
Grand Total	161,983.50	1,018,160.40	456,646.25	109,202.60	1,745,992.75

Section 7

i) Grantees' signatures

15-10-2010

ii) Comments of the Head of the Department/signature

Prof. C. Deepal Mathew
Head/Dept. of Biochemistry
& Molecular Biology
Faculty of Medicine
University of Colombo

iii) Head of the Institution's signature

DEAN
FACULTY OF MEDICINE
DATE 15.9.10

iv) Vice Chancellor's Signature

Vice-Chancellor
University of Colombo

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PHCOG MAG.: Research Article

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°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.

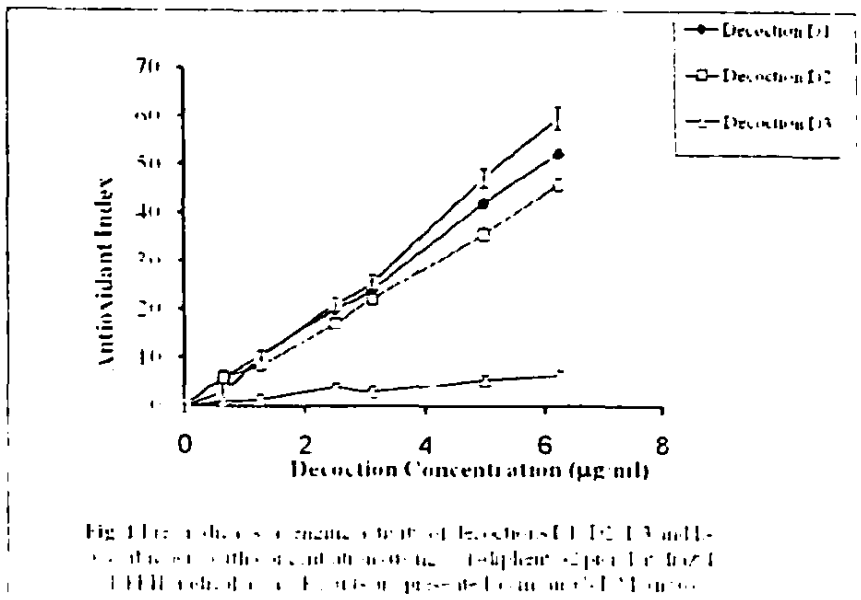
RESULTS

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.



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.

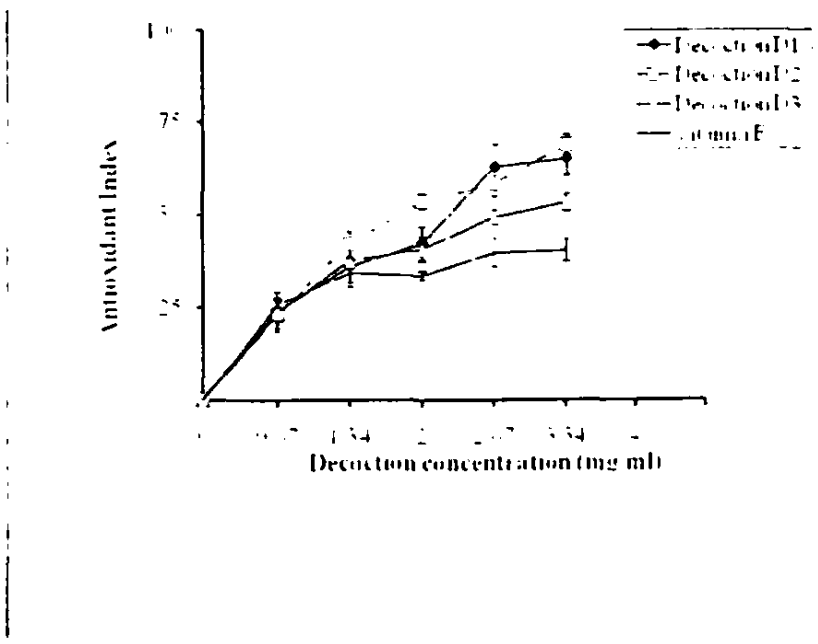


Fig. 2. Antioxidant Index of decoctions D1, D2, D3 and Vitamin E at different concentrations on Lipid peroxidation in egg yolk. Results areresented as mean ± S.E.M. (n=4).

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.

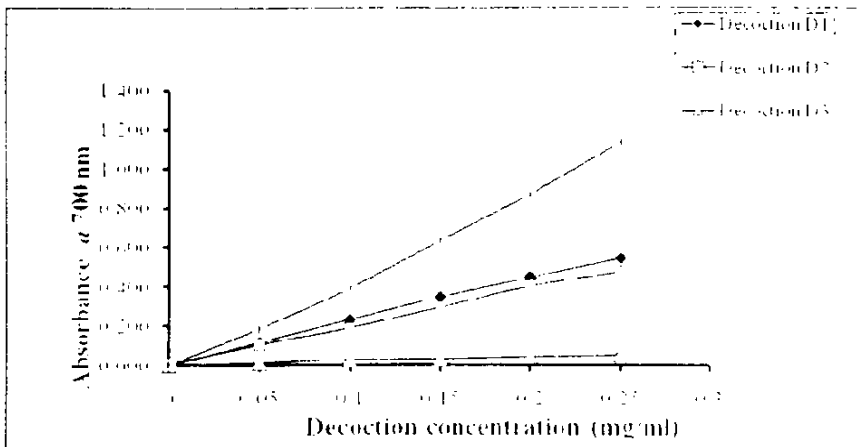


Fig.3. Total reduction capability of different concentrations of decoction D1, D2, D3 and ascorbic acid using spectrophotometric detection of the Fe^{3+}-Fe^{2+} transformation. Results are presented as mean ± SD (n=3)

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 (µg/ml)	% 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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01

From: Dr. Sheo Mohan Singh <icscc2010@isquareit.ac.in>
Subject: abstract accepted for poster presentation
To: "'indiwari kalhari'" <indikalha@yahoo.com>
Cc: sheomohan@yahoo.com
Date: Sunday, 22 August, 2010, 8:46 AM

Dear Kalhari,

We are pleased to inform you that your abstract titled "**Antiproliferative activity and induction of apoptosis by a decoction prepared from *Adenanthera pavonina* and *Thespesia populnea***" is accepted for **Poster** presentation at the "International Conference on Stem Cells and Cancer (ICSCC-2010): Proliferation, Differentiation and Apoptosis", 11-14 December 2010, Pune, India.

The exact date and time of presentation will be intimated separately.

We look forward to welcoming you here in December.

With best wishes,

Sheo

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02

Antioxidant and cytotoxic activities of a decoction prepared from *Adenanthera pavonina* (madatiya) *and Thespesia populnea* (gansuriya)

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The exploitation of plants for their medicinal potential and the phenomenon "stem to pill" has been a topic of much discussion. However, a very few of them are subjected to a comprehensive analysis. A decoction comprising *Adenanthera pavonina L.* and *Thespesia populnea L* is used to treat cancer by traditional physicians in Sri Lanka. Potential antioxidant and cytotoxic properties of the decoction were investigated in this study.

Lyophilized extract of the decoction was used to study the antioxidant activity and cytotoxicity. Antioxidant activity of the decoction was investigated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and nitric oxide radical scavenging assay. Larynx carcinoma (*Hep-2*) tumor cells were treated for 24 hours with different concentrations of the decoction at 37°C. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the antiproliferative activity of the decoction.

The mean (± SD) values for EC_{50} were 7.24 (± 0.495) and 14.02 (± 0.66) mg dm^{-3} for DPPH and nitric oxide radical scavenging activity respectively. The Mean (± SD) value for EC50 was 140.69 (± 12.57) mg dm^{-3} for MTT assay. These results provide the chemo preventive and therapeutic potential of the decoction containing *Adenanthera pavonina L.* and *Thespesia populnea L.*

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03

Comparison of cell viability of Black, Green and White tea (*Camellia sinensis*) manufactured in Sri Lanka

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Abstract

Dietary polyphenols have attracted a great deal of interest because of their perceived ability to act as highly effective chemo preventive and chemotherapeutic agents. Among dietary polyphenols, tea (*Camellia sinensis*) has drawn a major attention since it, is a source of compounds with antioxidative, antimicrobial, antimutagenic and anticarcinogenic properties. However, the cytotoxicity of tea produced in Sri Lanka is not well studied.

The objective of this study was to compare the cell viability among the above three tea extracts. The *Hep-2* cell lines (laryngeal carcinoma) were used for the cytotoxicity assays. The cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The *Hep-2* cells were treated at different concentrations of water extracts of black tea (200-1200 μg cm^{-3}), white tea (25-500 μg cm^{-3}) and green tea (12.5-800 μg cm^{-3}) for 24 hours. In all experiments negative control without any tea extracts and positive control with Camptothecin (5mM) was used.

The present results show that the tea extracts exert dose-dependent suppression of cell proliferation with MTT assay. The EC_{50} value for black tea, green and white tea were 683.21 ± 2.52 μg cm^{-3}, 58.00 ± 0.00 μg cm^{-3} and 355 ± 0.01 μg cm^{-3} respectively. This concludes that green tea produced in Sri Lanka has more cytotoxic properties against Hep-*2* cells followed by black tea and white tea based on the MTT assay. The percentage cell viability of the positive control was 35% to that of untreated cells.

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This work is supported by National Science Foundation grant No. RG/2005/HS 17.

CYTOTOXICITY ON *Hep 2* CELL LINE BY BLACK TEA EXTRACT (*Camellia sinensis*) AFTER REMOVAL OF ITS' POLYPHENOLIC FRACTION

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Abstract

Most of the studies on cancer preventive properties of tea (*Camellia sinensis*) have been carried out with black tea extracts or individual catechins (polyphenolic compounds) present in tea. However tea contains number of substances other than polyphenols. The objective of this study was to investigate, the antioxidant and cytotoxic properties of high grown black tea manufactured in Sri Lanka and to investigate the same properties after removal of polyphenols from the black tea.

Black tea extract (BT) was prepared by boiling tea leaves with distilled water and polyphenols were separated from tea extract using polyvinylpolypyrrolidone. Both extracts were tested for phenolic content by Folin-Ciocalteu's reagent. Caffeine and gallic acid present in black tea and polyphenol free tea extracts were quantified by HPLC. Free radical scavenging activity (DPPH assay) of black tea and polyphenol free tea were determined by DPPH assay. Ascorbic acid was used as the positive control.

The *Hep 2* cells were treated with BT or polyphenol free tea extracts at different concentrations (200-2000 μg cm^{-3}) for 24 hours. The cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and lactate dehydrogenase release (LDH). The morphological changes were observed under light microscopy.

Caffeine present in BT is similar to that of polyphenol free tea and the gallic acid content in polyphenol free tea was only 8.15% of the black tea extract. The EC_{50} value for DPPH assay was 56.81 ± 1.73 μg cm^{-3} (n=3), which is higher compared to the ascorbic acid. In the absence of polyphenols, no DPPH scavenging activity was shown with black tea over the concentrations investigated.

BT exerts dose-dependent suppression of cell proliferation with MTT assay and the EC_{50} was 683.21 ± 2.52 μg cm^{-3}. The EC_{50} value for LDH assay was 1.60 ± 0.31 mg cm^{-3} (n=8). The cell viability for polyphenol free tea (n=4) was 90-100% that of the control, as determined by MTT assay and the LDH release to the medium by *Hep* 2 cells was only 20-23% of the total LDH over the concentrations investigated. Changes in cell morphology were observed only with increasing concentration of BT, but not with polyphenol free tea.

The results clearly suggest that the principal components responsible for the antioxidant activity and cytotoxicity are polyphenolic substances present in black tea extract and without them no cytotoxicty is demonstrated.

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853/E2

Comparison of the antioxidant activity of Black, Green and White Tea

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Most of the studies on cancer preventive properties of tea (*Cammelia sinensis*) have been carried out with green tea, black tea extracts or individual catechins (polyphenolic compounds) present in tea. The composition of tea polyphenols differs for black tea, green tea and white tea because of the different degrees of fermentation during manufacture. The objective of this study is to compare the antioxidant activity of different types of tea related to their method of manufacture.

Different types of tea products were boiled with distilled water for 10 minutes to obtain the tea extracts. Total free radical scavenging activity (DPPH assay), reducing power and anti lipid peroxidation activity (TBARS assay) of black tea, green tea and white tea were investigated and compared with ascorbic acid and vitamin E. Further polyphenols were separated from tea, and assayed for DPPH radical scavenging activity to study the contribution of polyphenols to the free radical scavenging activity.

The EC_{50} values for black, green and white tea were; for DPPH assay 56.81 ± 1.73, 19.12 ± 0.08 and 34.11 ± 0.26 mg dm^{-3} respectively; for the lipid peroxidation assay, 2.79 ± 0.26 , 1.95 ± 0.11 and 1.275 ± 0.03 mg dm^{-3} respectively. In the absence of polyphenols, no DPPH scavenging activity was shown with black tea. The reducing power was highest with white tea followed by black and green tea.

For DPPH radical scavenging assay green tea shows the highest antioxidant activity. However, white tea shows the highest antioxidant activity for lipid peroxidation and measurement of reducing power assays.

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06

Study of the antioxidant activity of a traditional herbal oil used for cancer therapy in Sri Lanka.

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In Asian countries like Sri Lanka and India, there are well-developed traditional medical systems, which use various herbal preparations to treat cancer. These herbal formulations are prepared using a mixture of plant material according to traditional formulas. The therapeutic benefit of these medicinal preparations is often attributed to their antioxidant properties. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radicals or active oxygen scavengers those of which play a significant role in prevention and cure of cancer through elimination of carcinogens from the system. It has been shown that phenolic substances present in plants have major contribution for antioxidant activity.

The objective of this study was to investigate the antioxidant activity of a traditional herbal oil which is called " pranajeewa' which is used in certain types of cancer and other diseases in traditional medicine of Sri Lanka. Antioxidant activity of the oil was investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method, reducing the power and anti lipid peroxidation activity (TBARS assay). These values were compared with ascorbic acid and vitamin E. The phenol content was investigated by Folin-Ciocalteu method. The polyphenol content of the oil was 0.29 ± 0.09 % w/w galic acid equivalents. The EC_{50} value for DPPH radical scavenging activity of the oil was 79.84 ± 5.35 µl/ml. The EC_{50} value for TBARS assay of the oil was 19.24 ± 0.52 µl/ml.

The above findings indicate that ' pranajeewa' oil has free radical scavenging activity and lipid peroxidation activity, however the antioxidant properties are lower than ascorbic acid vitamin E.

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compounds of the plants used in the Sidha formulation and the mercury showed oxygen coupling. It is presumed that mercury is present as a caged compound which needs to be further established, through analysis and single crystal studies. The focus of the present paper is more on the invitro cytotoxicity and acute toxicity of the Sidha formulation and its material nature. The material composition as such is indicated by the SEM, TEM, FT-IR and thermogravimetric studies, which renders the compound as nontoxic as revealed through acute toxicity and invitro lymphocyte toxicity studies.

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Study on Antiproliferative Activity of a Selected Decoction used for Cancer Therapy in Sri Lanka

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Although a variety of medicinal plants are abundantly used in traditional drug prescriptions as an alternative treatment for cancer in Sri Lanka, a very few of them have been subjected to analysis of their constituents and cytotoxicity. This study was carried out to investigate the antioxidant, antiproliferative and cytotoxic properties of a selected decoction used in cancer therapy using Hep-2 cells.

The decoction was consisted with Boerhavia diffusa L (root), Crateva adensonii (root), Toddalia asiatica (L.) (root), Anacyclus pyrethrum DC (root)., Bombax ceiba (dried gum) and Ricinus communis L (root). Water extract of decoction was prepared following the traditional method used in Sri Lanka.

Phenolic content and the gallic acid present in the decoction were quantified. Total free radical scavenging activity was investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and compared with Vitamin C. Hep2 cells were exposed at different concentrations of the decoctions for 24 hours. Cytotoxicity and cell viability were assayed by lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays, and protein synthesis. Morphological changes were observed by fluorescence microscopy.

The total phenolic and gallic acid present in the decoction were 14.42 ± 0.67 % w/w gallic acid equivalents and 1.02 ± 0.60 mg g^{-1} respectively. The Ec_{50} value for DPPH radical scavenging activity was 40.18 ± 3.7 µg cm^{-3}. The IC_{50} values for MTT and LDH assays were 1.06 ± 0.002 and 1.23 ± 0.130 mg cm^{-3} respectively. A dose dependent decrease in protein synthesis in Hep-2 cells was observed with increasing the concentration and the decrease was 25% compared to the negative control at a concentration of 1.0 mg cm^{-3} Rounding and detachment of the cells were observed with the increasing the concentration of the decoction.

Decoction investigated in this study shows lower free radical scavenging capacity compared to vitamin C, however it shows antiproliferative and cytotoxicity toward Hep2 cells over the concentration investigated.

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Characterization of biological activity of *Flueggea leucopyrus* Willd. (katupila)

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Flueggea leucopyrus Willd. is a medicinal plant used for the treatment of many diseases including cancer in the Ayurvedic system of medicine. The present study was carried out to investigate the cytotoxic and antioxidant properties of the aqueous extracts of leaves and stem of the plant. Brine shrimp bioassay was used to study the cytotoxic properties of the plant. The crude extracts of leaves and stem did not show cytotoxic activity (IC_{50} >30 μg ml^{-1}) in the brine shrimp bioassay.

The aqueous extracts of leaves and stem were investigated for their total phenolic content and antioxidant properties. The leaves and stem extract contained a total phenolic content of 18.12% and 12.08% w/w of gallic acid equivalents respectively. The free radical scavenging activity (DPPH assay) of aqueous extracts of leaves and stem were investigated and compared with ascorbic acid which was used as a reference standard. The DPPH free radical scavenging activity of the leaves and stem extracts showed IC_{50} values of 8.08 and 17.56 μg ml^{-1} respectively. The IC_{50} value of ascorbic acid was 5.29 μg ml^{-1}. The Nitric oxide radical scavenging activity of the aqueous extract of the leaves and stem were also studied. The leaves showed concentration dependent NO radical scavenging activity at concentrations less than 5 μg ml^{-1}, giving IC_{50} value between 1.7-2.1 μg ml^{-1}. The stem extract did not show any dose dependent relationship towards NO radical scavenging activity. According to the phytochemical screening results, alkaloids, leucoanthocyanins and tannins of the pyrogallol type are present in both the leaves and stem of the plant. The results obtained in the present study indicate that aqueous extracts of the aerial parts of *Flueggea leucopyrus* Willd. is a potential source of natural antioxidants. Antioxidants have also been reviewed for their possible role in the prevention of cancer. Hence the results obtained from this study could be used in the rationalization of ethnomedical use of the plant.

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Anti-oxidative activity of *Pleurotus cystidiosus*

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Antioxidants have been isolated from plants and fungi and they are mostly polyphenols and flavonoids. Ji-Kai Liu and his coworkers have reported the DPPH radical scavenging activities of ten natural *p*-Terphenyl derivatives obtained from three mushrooms indigenous to China. Curtisians A-D isolated from *Paxillus crutissii* have shown 10-20 times more antioxidant activities than that of vitamin E against lipid peroxidation. Betulinan A and B obtained from *Lenzites betulina* and hispidine derivatives obtained from *Phellinus linteus* mycelial culture broth have reported to have strong antioxidant activities. Pleuran (β-1,3-D glucan) isolated from *Pleurotus ostreatus* has also shown antioxidant activity. This study was carried out to investigate the antioxidant activities of the edible mushroom *Pleurotus cystidiosus*, commonly known as Abalone.

Compounds in fresh *P. cystidiosus* mushroom were extracted into acetone (A), dichloromethane (D) and hexane (H). Freeze dried extract A was fractionated using solvent extraction method to obtain A1, A2, A3 and A4 fractions. Fraction A4 was further separated into A4-1, A4-2 and A4-3 fractions using a reverse phase column. DPPH radical scavenging activity and nitric oxide radical scavenging activity were assayed for extract A and A4, A4-1, A4-2 & A4-3 fractions. All experiments were performed in triplicates. The respective EC_{50} values obtained for DPPH radical scavenging assay were 1.12 mg cm^{-3}, 1.13 mg cm^{-3}, 0.87 mg cm^{-3}, 0.81 mg cm^{-3} & 0.82 mg cm^{-3} and EC_{50} values of nitric oxide radical scavenging assay were 4.81 mg cm^{-3}, 3.82 mg cm^{-3}, 5.38 mg cm^{-3}, 0.87 mg cm^{-3} & 0.61 mg cm^{-3}. EC_{50} value obtained for ascorbic acid in DPPH radical scavenging assay was 44.57 μg cm^{-3}.

The antioxidant activity shown by the DPPH radical scavenging assay and nitric oxide radical scavenging assay indicates that fractions A4-2 and A4-3 to have the highest activity. We conclude that the polar aqueous fractions of *P. cystidiosus* contain the compounds having antioxidant activities and there exists a possibility to use such extracts as a food additive.

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10 **Study on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity in relation to the phenolic and gallic acid content in four medicinal plants used for cancer therapy in Sri Lanka**

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Antioxidants have an important role in preventing a variety of diseases including cancer. It has been shown that phenolic substances present in plants have major contribution for their antioxidant activity. The objective of this study was to investigate, the antioxidant activity in relation to the polyphenolic and gallic acid contents in four traditional medicinal plants used by traditional medical practitioners in Sri Lanka for cancer therapy.

The total phenolic content present in water extract of *Smilax glabra* (Cheena Ala; root), *Bombax ceiba* (ela imbul; gum), *Anacyclus pyrethrum* (Akkarapatta; root) and *Hemidesmus indicus* (Iramusu; root) was determined by Folin-Ciocalteu method. Caffeine and gallic acid were quantified by high performance liquids chromatography (HPLC). Total free radical scavenging activity of each ingredient was investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and the values were compared with phenolic and gallic acid present in each plant.

The polyphenol content of *Bombax ceiba*, *Anacyllus pyrethrum*, *Hemidesmus indicus* and *Smilax glabra*, were 32.57 ± 5.04 %, 30.98 ± 2.97 14.52 ± 1.13 and 19.43 ± 2.89 % w/w gallic acid equivalents respectively. Detectable levels of gallic acid were present only in *Bombax ceiba* (1.46 mg g^{-1}) and *Smilax glabra,* (0.94 mg g^{-1}). The EC_{50} values for DPPH radical scavenging activity for *Bombax ceiba*, *Anacyllus pyrethrum Hemidesmus indicus* and *Smilax glabra* were 15.47±1.80, 15.01±0.82, 46.78±16.03 and 35.67±0.64 µg cm^{-3}. The mean values of EC_{50} (y) for DPPH radical scavenging activity were correlated with total phenolics (x) present in plant extracts ($y = -35.417x + 1428$; $R^2 = 0.9887$), for all plant ingredients used in this study.

The above findings suggest that phenolic substances present in the four plants, contribute to their free radical scavenging activity in the presence or absence of endogenous gallic acid.

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Total phenolic and gallic acid content strongly associate with antioxidant and cytotoxic properties of three traditional decoctions used for the treatment of cancer in Sri Lanka

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Abstract

Herbal medicines have a vital role in the prevention and treatment of cancer. Many plant-based treatments are being prescribed for cancer treatment by traditional medical practitioners of Sri Lanka. Three such decoctions which composed of *Terminalia bellerica, Terminalia chebula, Phyllanthus emblica and detoxified Commiphora mukul* (D1), *Terminalia bellerica, Terminalia chebula, Phyllanthus emblica, detoxified Commiphora mukul, Smilax china and Nigella sativa* (D2) and *Munronia Pumila, Azadirachta indica, Solanum surattense, Solanum xanthocarpum, Rubia cordifolia, Picrorhiza kurroa, Trichosanthes cucumerina and Pterocarpus santalinus* (D3) were selected to investigate their total phenolic contents, antioxidant properties and potential cytotoxic activities. The total phenolic contents of D1,D2 and D3 were 37% , 30% and 6% w/w respectively. Gallic acid present in each decoction was analysed by HPLC and the mean (SD) values were 6.97 (0.03), 5.89 (0.14) and 1.14 (0.00)% for D1,D2 and D3 respectively. Total free radical scavenging activity (DPPH assay), reducing power and antilipid peroxidation activity (TBARS assay) of each decoction were investigated and the values were compared with ascorbic acid and vitamin E. The EC_{50} value for DPPH assay with decoction D3 showed 20 times higher value than that of D1 and D2. Small but significant increase in EC_{50} value was shown with D3 compared to that of D1 and D2 with anti lipid peroxidation assay. Similar results were obtained for reducing power for D1 and D2 however the values are lower than that of ascorbic acid over the concentration range of 50-300ug/ml. The reducing power obtained for both decoctions D1 and D2 at a concentration of 200 μg/ml was equivalent to that of 100μg/ml of ascorbic acid and it was negligible with D3. The MTT assay and LDH assay were used to investigate antiproliferative and cytotoxic activities of these decoctions against the human Rhabdomyosarcoma (RD) cells after exposure to 24 hours. The percentage LDH release to that of control was 93.4% and 68.8 % at a concentration of 150 μg/ml for D1 and D2 respectively. The percentage cell viability to that of control was 10.3% and 41.5% at a concentration of 150 μg/ml for D1 and D2 respectively. Decoction D3 did not show considerable cytotoxic effect Present study indicates that antiproliferative and cytotoxic activities in Rhabdomyosarcoma (RD) cells by these decoctions are strongly associated with their total phenolic and gallic acid content

Keywords: Total phenolic content Gallic acid, Antioxidant activity, Antiproliferative activity, Cancer, Cytotoxicity, Herbal decoctions,.

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