Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Anti-hepatocarcinogenic and Anti-oxidant Effects of Mangrove Plant *Scyphiphora hydrophyllacea*

Sameera R. Samarakoon, Chanthirika Shanmuganathan, Meran K. Ediriweera, Poorna Piyathilaka, Kamani H. Tennekoon, Ira Thabrew, Prasanna Galhena[1], E Dilip De Silva

Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, 90, Cumaratunga Mawatha, Colombo 3, Sri Lanka, [1]Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, Thalagolla Road, Ragama, Sri Lanka

Submitted: 10-11-2015 Revised: 07-12-2015 Published: 07-04-2017

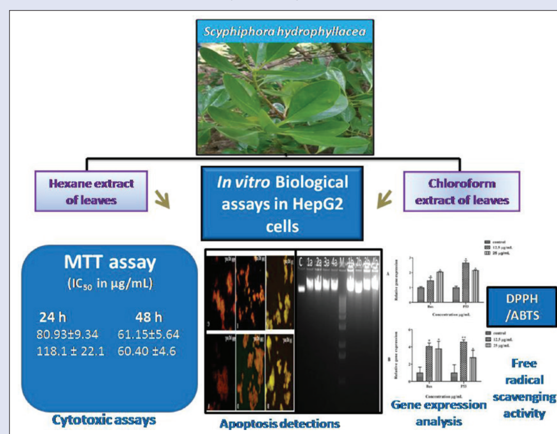
ABSTRACT

Context: *Scyphiphora hydrophyllacea* is a shrub mangrove plant of the family Rubiaceae and not yet been studied for anti-hepatocarcinogenic effects. **Objectives:** We investigated possible *in vitro* anti-hepatocarcinogenic and antioxidant properties of *S. hydrophyllacea*. **Materials and Methods:** Dried leaves of *S. hydrophyllacea* were sequentially extracted into hexane, chloroform, ethyl acetate, and methanol and tested for cytotoxicity on HepG2 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and sulforhodamine B assays, and for antioxidant activities by the free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assays. Total phenolic and flavonoid contents were estimated in all four extracts. The hexane and chloroform extracts were tested for pro-apoptotic properties in HepG2 cells, and bioactive components were identified by gas chromatography–mass spectrometry (GC-MS) analysis. **Results:** The hexane and chloroform extracts showed dose-dependent and time-dependent cytotoxic effects. Morphological changes observed under fluorescence microscope related to apoptosis, and significant ($P < 0.001$) increases in caspase 3 and 9 levels were observed in hexane and chloroform extract-treated cells. Slight DNA fragmentation was observed only in response to the chloroform extract. mRNA expressions of *p53* and *Bax* were significantly upregulated by low doses of hexane and chloroform extracts. Highest antioxidant activity was observed in the methanol extract. GC-MS profiles identified 24 and four major compounds in the hexane and chloroform extracts, respectively. These included some known anticancer compounds such as lupeol. **Conclusion:** Cytotoxicity, antioxidant effects, and apoptosis-related changes exerted by hexane and chloroform extracts of *S. hydrophyllacea* concluded that these two extracts are good source for isolation of possible anticarcinogenic compounds.

Key words: *Scyphiphora hydrophyllacea*, mangrove, HepG2 cells, antioxidant, apoptosis

SUMMARY

- The hexane and chloroform extracts of *Scyphiphora hydrophyllacea* showed dose-dependent and time-dependent cytotoxic effects.
- Morphological changes related to apoptosis and significant ($P < 0.001$) increases in caspase 3 and 9 levels were observed in hexane and chloroform extract-treated cells.
- mRNA expressions of *p53* and *Bax* were significantly upregulated by low doses of hexane and chloroform extracts.
- Highest antioxidant activity was observed in the methanol extract.
- GC-MS profiles identified 24 and four major compounds in the hexane and chloroform extracts, respectively.



Abbreviation used: DPPH: 1,1-diphenyl-2-picryl-hydrazyl, ABTS: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, GC-MS: gas chromatography–mass spectrometry, DNA: deoxyribonucleic acid, HCC: Hepatocellular carcinoma, GAE: gallic acid equivalents, SRB: sulforhodamine B, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AO/EB: acridine orange/ethidium bromide, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, IC_{50}: half maximal inhibitory concentration; QE: quercetin equivalents, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract, ME: methanolic extract, TPC: total polyphenol content, TFC: total flavonoid content, ANOVA: Analysis of variance

Correspondence:
Dr. Sameera R. Samarakoon,
Scientist, Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo, 90 Cumarathunga Munidasa Mawatha, Colombo 3, Sri Lanka,
E-mail: sam@ibmbb.cmb.ac.lk
DOI: 10.4103/0973-1296.203989

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Hepatocellular carcinoma (HCC) has been rated as the fifth most commonly occurring cancer and the third most common cause of cancer-related deaths in the world. There are several treatment options available for HCC including liver transplantation, surgical resections, radiofrequency ablations, transarterial chemomobilization, and radiomobilization.[1] Locoregional therapies and surgical treatments for HCC have advanced in the past few years, but the recurrent HCC has remained a problem faced by clinicians worldwide.[2] Chemotherapy

This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Samarakoon SR, Shanmuganathan C, Ediriweera MK, Piyathilaka P, Tennekoon KH, Thabrew I, Galhena P, De Silva ED. Anti-hepatocarcinogenic and anti-oxidant effects of mangrove plant *Scyphiphora hydrophyllacea*. Phcog Mag 2017;13:76-83.

 © 2017 Pharmacognosy Magazine | Published by Wolters Kluwer - Medknow

and radiotherapy treatments for HCC cause survival of patients with severe adverse effects including cirrhosis.[3] Therefore, it is essential to search for new anticancer agents for HCC with fewer adverse effects. Many anticancer drugs now available in the market and drugs that are currently under clinical trials in humans are phytochemicals.[4]

During the past few years many have researched the presence of therapeutically useful compounds in mangrove plants. Most of the mangroves are woody plants growing along seashores in tropical and subtropical areas and contain plenty of chemical constituents with potential medicinal values.[5,6] *Scyphiphora hydrophyllacea* is one of the shrub mangrove plants and grows to about 3 m in height. It belongs to the family Rubiaceae. It is distributed from Southeast Asia to New Caledonia.[7] *S. hydrophyllacea* is reported to contain flavonoids, terpinoids, and iridoids as major phytochemicals. Two noriridoids and iridoids have also been isolated from this plant.[8] There are no reports on possible cytotoxic, apoptic, and antioxidant effects of this plant on HepG2, human hepatoma cells.

Therefore, this study was carried out with the objectives of assessing (a) possible cytotoxic and apoptotic effects of *S. hydrophyllacea* grown in Sri Lanka on HepG2 cells and (b) to identify the active phytochemical constituents with anticancer activity from the leaves of this plant.

MATERIALS AND METHODS

Chemicals

All the chemicals and cell culture reagents used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and American Type Culture Collection, respectively.

Plant material

Healthy, mature *S. hydrophyllacea* leaves were collected from the mangroves park, Kadolkele, Negombo, in the Western Province of Sri Lanka (Lat. 7°11’54.03” Long. 79°50’30.94”), during April 2013 and voucher specimen (S-11) authenticated by Mr. W.A. Sumanadasa, of the National Aquatic Resources Research and Development Agency, Negombo, Sri Lanka has been deposited in the Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

Preparation of plant extracts

Leaves of the plants were dried at room temperature and ground into powder using an electrical grinder. Sixty grams of ground plant material were extracted sequentially in to hexane, chloroform, ethyl acetate, and methanol at room temperature. Subsequently, each extract was filtered and concentrated in a rotary evaporator (Rotavapor R-/; BÜCHI Labortechnik AG, Flawil, Switzerland) and stored at 20°C until used.

Phytochemical investigations

Total phenolic content

The total phenolic content of all four extracts was measured by the Folin Ciocalteau reagent as previously reported.[9] Gallic acid was used as the standard and results expressed as milligrams of gallic acid equivalents (GAE) per gram extract (dry weight), that is, mg GAE/g extract (dry weight).

Total flavonoid content

Dowd method adapted from Meda *et al*., 2005[10] was used to determine the total flavonoid content. Methanol (5 mL) containing 2% $AlCl_3$ was added to 1 mL of extract diluted in methanol and incubated for 1 h at room temperature. Then, the absorbance was measured at 415 nm using the Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Total flavonoid content was expressed as quercetin equivalents per extract dry weight (mg/g).

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl radical scavenging ability assay

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical was used to determine the radical scavenging activities of all four extracts as described by Mothana *et al*.,[11] with slight modifications. The reaction mixture was made by adding 100 µL of the extract diluted in methanol, 25 µL of 1 mM DPPH and 75 µL of methanol in to the 96-well plate. After incubating the plate for 30 min at 37°C, decline in the absorbance was observed at 517 nm by using Microplate Reader (Synergy HT Multi-Mode). Ascorbic acid was used as the standard. Scavenging activity was determined using following equation.

Scavenging activity (%) = {(Ac – As)/Ac} 100, where Ac and As are the absorbance of control and test samples, respectively. The data are presented are mean ± SD (n = 3) and expressed as IC_{50} (half maximal inhibitory concentration) value.

2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid

2,2’-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) free radical scavenging activity of the extracts was determined by the method described by Re *et al.*[12] ABTS working solution was prepared by mixing equal volumes of 7 mM ABTS stock solution and 2.4 mM potassium persulfate stock solution and the mixture was kept in the dark for 5 h at room temperature. After diluting the mixture with phosphate-buffer saline (PBS, pH 7.4), absorbance was read at 734 nm. Samples diluted in PBS were further mixed with the solution of the radical cation $ABTS^+$ and the decrease in the absorbance was noted after 10 min at 734 nm. Trolox (concentrations of 1.1, 1.7, 2.3, 2.9, and 3.5 µg/mL) was used as the positive reference. The data are presented are mean ± SD (n = 3) and expressed as IC_{50} (The half maximal inhibitory concentration) value.

Cell culture maintenance

HepG2 cells were cultured in a humidified environment (37°C, 95% air, 5% CO_2) in complete culture medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 50 IU/mL penicillin and 50 µg/mL streptomycin.

Cells were trypsinized and seeded (5×10^3 cells/well) in 96-well cell culture plates to study the cytotoxicity of extracts or, in appropriate concentration, to set up assays for other studies.

Assessment of cytotoxicity

Cytotoxicity of the solvent extracts on HepG2 cells was evaluated using sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays as previously described by Samarakoon *et al.*[13] Cells were incubated for 4, 24, and 48 h with different concentrations of the extracts (10, 20, 50,100, and 200 µg/mL). Cells were subjected to either MTT or SRB assays at the end of the incubation.

Effect on apoptosis

Hexane and chloroform extracts that demonstrated highest cytotoxicity were chosen to study pro-apoptotic effects. Effects of the active extracts on apoptosis were evaluated by (a) analysis of morphological changes related to apoptosis, (b) DNA fragmentation analysis, and (c) caspase 3 and 9 activities as described in the following sections.

Analysis of morphological changes by fluorescent microscopy

Morphological changes of the cells treated with hexane and chloroform extracts and control (untreated) cells were investigated under fluorescence microscope after staining with acridine orange/ethidium bromide (AO/EB) as described by us with slight modification.[14] Briefly, cells were seeded at a

concentration of 2×10^5 cells/mL in 24-well culture plates on cell culture-treated cover clips. The cells were exposed to different concentrations of the hexane and chloroform extracts (0–400 μg/mL) for 24 h. Changes in the nuclei of cells after AO/EB staining were observed under a fluorescence microscope at 24 h postincubation (Olympus, BX51TRF, Japan).

Evaluation of caspase 3 and caspase 9 activities

HepG2 cells (4×10^5 cells/mL) were incubated with different concentrations of either the hexane (12.5, 25, and 50 μg/mL) or the chloroform (25, 50, and 100 μg/mL) extract for 24 h to study the effect on caspases. Caspase 3 and caspase 9 activities were measured using commercially available reagents (GenScript, Piscataway, USA) according to the manufacturer's instructions.

DNA fragmentation assay

HepG2 cells (2×10^5 cells/ mL) were treated with 200 and 400 μg/mL of either the hexane or the chloroform extract for 24 h and harvested by trypsinization and centrifugation. DNA fragmentation assay was carried out as described by Samarakoon *et al.*[15]

Evaluation of mRNA expression of p53 and Bax by real-time quantitative polymerase chain reaction

HepG2 cells were seeded in the 25 cm^2 cell culture flasks (4×10^5 cells/mL) and allowed to attach overnight. The cells were then treated with different concentration of either hexane (12.5, 25 μg/mL) or chloroform (25, 50 μg/mL) extracts for 24 h. Following incubation, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. An equal amount of total RNA from each sample was then reverse transcribed into cDNA using random primers. cDNA was mixed with 1 μL each of following gene-specific primers (0.2 mM): (i) Bax forward primer (5'- TCCAGGATCGAGCAGGGCGAA-3') and reverse primer (5'- CGATGCGCTTGAGACACTCGCT-3'), (ii) p53 forward primer (5'- TCTGGCCCCTCCTCAGCATCTT-3') and reverse primer (5'- TTGGGCAGTGCTCGCTTAGTGC-3'), and (iii) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer (5'- GGCATTGCCCTCAACGACCAC-3') and reverse primer (5'-ACATGACAAGGTGCGGCTCCCTA-3'). MESA Green qPCR mastermix Plus for SYBR assay (Eurogentec, Seraing, Liège, Belgium) was used to carry out real-time quantitative polymerase chain reaction. Reaction mixture was amplified by 40 cycles (denaturation, 95°C for 30 s; annealing, 58°C for 1 min; and extension, 72°C for 1 min) using an Mx 3000p Real-Time PCR System (Stratagene, La Jolla, CA, USA). Gene expression was quantified using the $2^{(-\Delta\Delta Ct)}$ method.[16]

Gas chromatography–mass spectrometry analysis of hexane and chloroform crude extracts

Phytochemical investigations of the hexane and chloroform crude extracts were carried out using Agilent gas chromatography-mass spectrometry (GC-MS) machine (Model 7890 GC, 5975-C MS; Agilent, Palo Alto, USA). The ionization voltage was 70 eV and the injector and detector temperatures were 260 and 320°C, respectively. The capillary column used was J&W DB 5 MS (30 m in length, 250 μm internal diameter, and 0.25 μm thickness). The oven temperature started at 110°C (isothermal for 5 min), and was ramped up to 280°C at 20°C/min (isothermal for 1 min) and up to 320°C at 20°C/min (isothermal for 5 min). Helium was used as the carrier gas at a flow rate of 1.5 mL/min with an injector volume of 1 μL with splitless mode. Prior to the injection, hexane crude extract was dissolved in hexane (1 mg/mL) and filtered through 0.2 μm syringe. NIST library search program was used to compare the mass spectras of compounds identified.

Statistical analysis

All the experiments were carried out in triplicate and results were expressed as mean ± SD (standard deviation) of at least three independent experiments. Graph Pad Prism (version 5.0; Graphpad Software, La Jolla, CA, USA) was used to perform the statistical analyses. One-way analysis of variance (ANOVA) followed by multiple comparisons by the Dunnett's test was used to assess the gene expression analysis and caspase activity. $P < 0.05$ was considered as statistically significant.

Results

Determination of total phenolic, flavanoid content, and free radical scavenging activities

As evident from [Table 1], total phenolic content was highest in the ethyl acetate extract, followed by the chloroform extract. Methanolic extract showed the highest flavanoid content followed by hexane, ethyl acetate, and chloroform extracts.

Both DPPH and ABST assays showed that the methanolic extract has the highest free radical scavenging activity. The lowest free radical scavenging activity was in the ethyl acetate extract as measured by the DPPH assay and in the hexane extract as measured by the ABTS assay. Both assays showed a moderate activity in the chloroform extract.

Cell viability

We examined the inhibitory effect of *S. hydrophyllacea* extracts on the growth of HepG2 cells using MTT and SRB assays for 4, 24, and 48 h. Hexane and chloroform extracts inhibited cell growth in a concentration-

Table 1: Total polyphenol and flavonoids contents and DPPH/ABTS free radical scavenging activity of four solvent extracts

Extract	TPC mg GAE/g	TFC mg QE/g	DPPH (IC_{50} μg/mL)	ABTS (IC_{50} μg/mL)
HE	53.90 ± 2.15	5.34 ± 1.71	254.90 ± 12.10	233.40 ± 36.10
CE	211.6 ± 12.30	0.46 ± 0.98	77.77 ± 3.787	63.97 ± 3.39
EAE	436.81 ± 15.40	5.49 ± 0.99	343.10 ± 18.34	157.20 ± 29.81
ME	166.20 ± 11.30	12.61 ± 0.15	22.18 ± 1.32	21.86 ± 2.57
Trolox			3.42 ± 1.40	2.90 ± 1.63

ABTS: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, DPPH: 1,1-diphenyl-2-picryl-hydrazy, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract, ME: methanolic extract, TPC: total polyphenol content, TFC: total flavonoid content. Trolox was used as the positive reference for DPPH and ABTS assays. Results are expressed as the mean ± SD of three parallel measurements. GAE: gallic acid equivalents, QE: quercetin equivalents.

Table 2: The *in vitro* cytotoxic activities of the four solvent extracts of *S. hydropyllacea* leaves

	SRB assay			MTT assay		
Time (h)	4	24	48	4	24	48
Hexane	>1000	82.52 ± 12.46	62.16 ± 11.23	> 1000	80.93 ± 9.34	61.15 ± 5.64
Chloroform	>1000	111.8 ± 21.59	63.01 ± 8.97	> 1000	118.10 ± 22.12	60.40 ±4.57
Ehtyl acetate	>1000	528.5 ± 32.54	651 ± 23.67	> 1000	639.00 ± 15.63	600.40 ± 18.43
Methanol	>1000	791.7 ± 21.67	>1000	>1000	732.90 ± 43.45	>1000
Paclitaxel*	28.88 ± 2.46	6.3 ± 1.29	2.86 ± 0.38	26.68 ± 3.21	5.35 ±2.91	2.99 ± 0.21

IC_{50} values are expressed in μg/mL. MTT = 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; SRB = sulforhodamine B

*Paclitaxel as positive control.

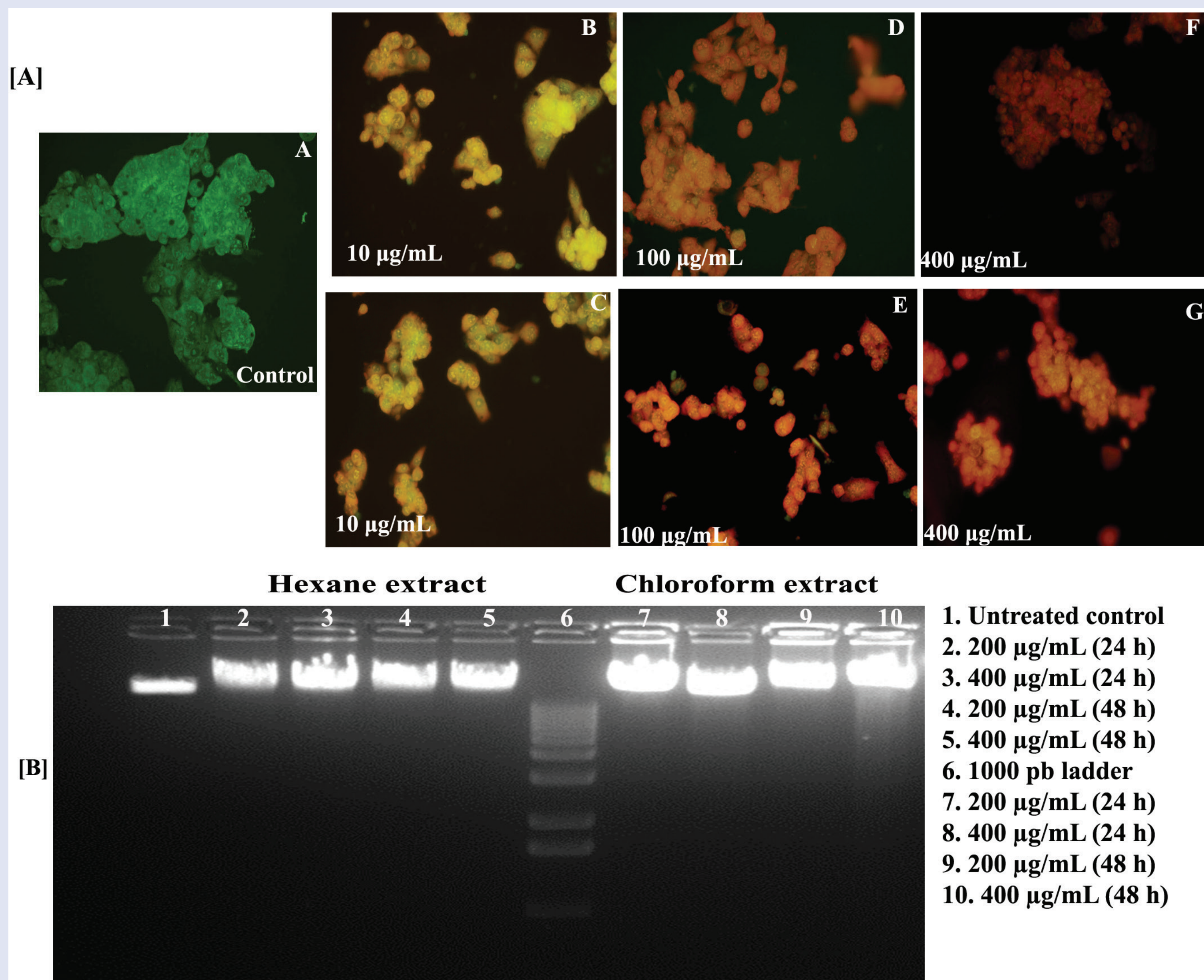


Figure 1: Morphological changes related to apoptosis observed under fluorescent microscope and DNA fragmentation analysis. [A] HepG2 cells treated with either the hexane or the chloroform extract for 24 h stained with acridine orange/ethidium bromide (AO/EB). A Control cells; B, D, F cells treated with 10, 100, 400 μg/mL of the hexane extract, respectively. C, E, G cells treated with 10, 100, 400 μg/mL of the chloroform extract, respectively. [B] DNA fragmentation in HepG2 cells following treatment with hexane and chloroform extracts analyzed by agarose gel electrophoresis.

dependent and time-dependent manner. The results are shown in Table 2. The IC_{50} value obtained for all the plant extracts against HepG2 cell was greater than 2 mg/mL at 4 h after incubation and hence considered non cytotoxic. However, the hexane and chloroform extracts exhibited significant cytotoxic effects with the IC_{50} values of 82.52 ± 12.456 (hexane) and 111.8 ± 21.587 (chloroform) μg/mL for SRB assay and 80.93 ± 9.343 (hexane) and 118.1 ± 22.123 (chloroform) μg/mL for MTT assay at 24 h after incubation. Ethyl acetate and methanol extracts showed less cytotoxicity to HepG2 cell (>500 μg/mL). Exposure to the hexane and chloroform extracts for 48 h enhanced the cytotoxic effects with the IC_{50} values of 62.16 ± 11.23 (hexane) and 63.01 ± 8.965 (chloroform) μg/mL for SRB assay and 61.15 ± 5.643 (hexane) and 60.40 ± 4.567 (chloroform) μg/mL for MTT assay. Further investigations were carried out to evaluate whether apoptosis was the mechanism responsible for the cytotoxic effects of hexane and chloroform extracts in HepG2 cells.

Fluorescent microscopy observations to evaluate apoptotic effects

Fluorescent microscopy observations of cells revealed that significant morphological changes related to apoptosis had occurred in the cells treated with the hexane and chloroform extracts when compared with control cells [Figure 1A]. Cells stained uniformly green by acridine orange represents untreated control cells, whereas cells appear in yellow color represent early apoptotic cells, and cells appear in reddish stained represent late apoptotic cells.

Caspase 3 and caspase 9 activities

As shown in Figure 2, caspase-3 and caspase-9 activity in HepG2 cells significantly ($P < 0.001$) increased in response to the hexane extract at lower doses (12.5 μg/mL), whereas the chloroform extract increased the activation of both the caspases at higher dose (50 μg/mL). Since expression of caspases is a characteristic feature in early apoptotic cells,

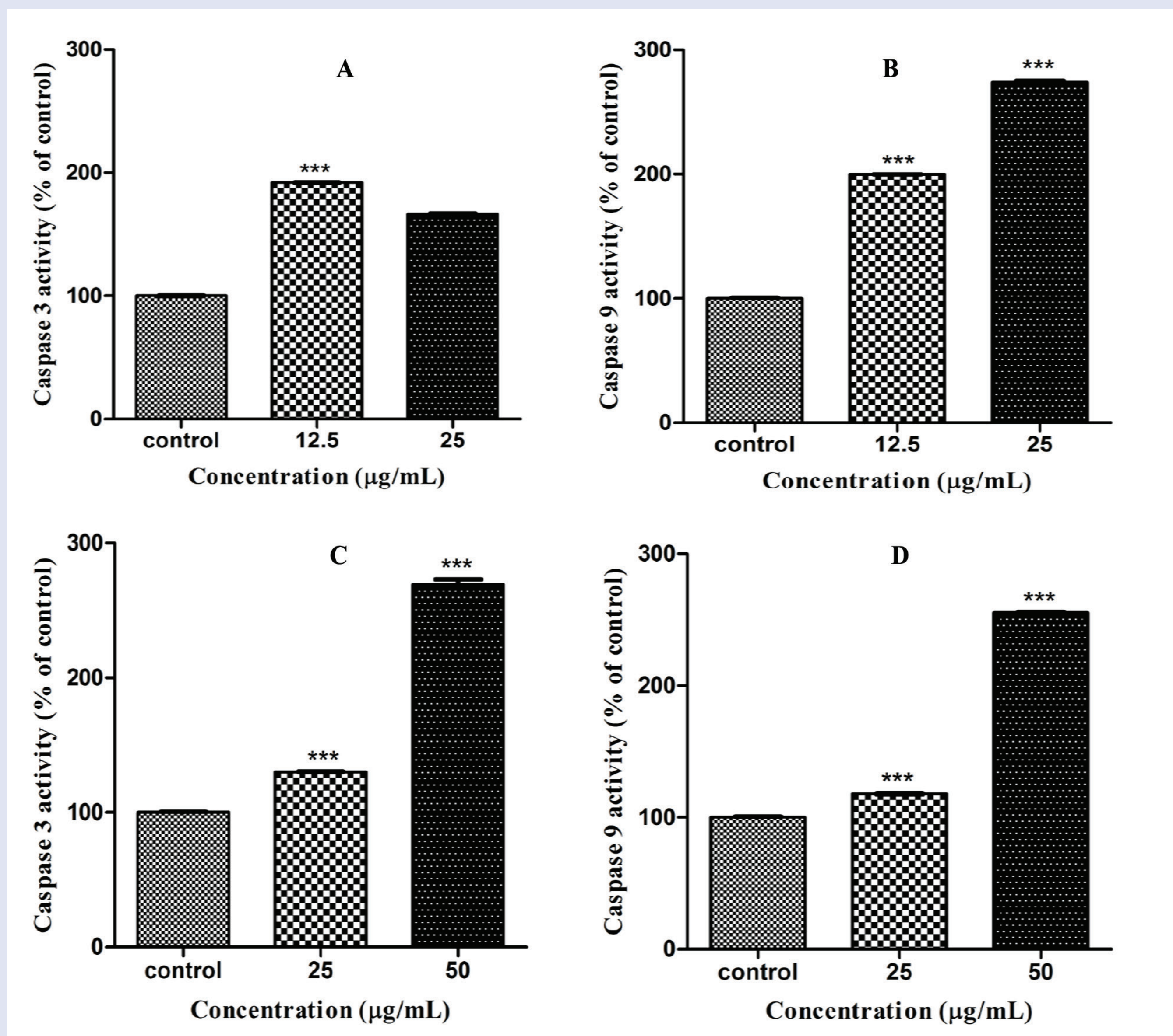


Figure 2: Activation of caspase 3 and caspase 9 in HepG2 cells 24 h after treatment with the hexane and chloroform extracts. Caspase 3 (A), caspase 9(B) activity of hexane extract treated cells. Caspase 3 (C), caspase 9 (D) activity of chloroform extract treated cells.****P*<0.001.

doses less than IC_{50} values were selected for the present experiment at 24 h after incubation in order to avoid late apoptotic changes in the cells.

DNA fragmentation

Slight DNA fragmentation was observed following exposure of HepG2 cells to the chloroform extract for 24 and 48 h, whereas no DNA fragmentation was evident in cells treated with the hexane extract for 24 and 48 h [Figure 2B].

Effect of S. hydrophyllacea on Bax and p53 gene expression in HepG2 cells

In the present study, a dose-dependent significant upregulation of pro-apoptotic *Bax* and *p53* were observed in both hexane and chloroform extract-treated HepG2 cells at 24 h after incubation suggesting activation of apoptosis via a p53-related mechanism [Figure 3]. However, p53 expression was slightly reduced at high doses (25 μg/mL hexane and 50 μg/mL chloroform extracts) of hexane and chloroform extracts than the expression level at the low dose (12.5 μg/mL hexane and 25 μg/mL chloroform extracts).

GC/MS analysis

GC-MS analysis of the hexane and chloroform extracts of the leaves of *S. hydrophyllacea* tentatively identified 28 major lipophilic compounds including sterols, fatty acids, long-chain hydrocarbons, and an unknown compound [Tables 3].

DISCUSSION

Much attention has been focused on exploiting the cytotoxic effects of natural compounds and plant-based remedies to discover novel and effective treatment modalities for different types of cancers including

Table 3: Major compounds in the hexane and chloroform extracts of *Scyphiphora hydrophyllacea* tentatively identified by GC-MS analysis

Hexane extract			Chloroform extract		
Retention Time (min)	Area (%)	Name of the Compound	Retention Time (min)	Area (%)	Name of the Compound
5.407	0.91	Hexadecane	23.22	3.10	Decahydro-8a-ethyl-1, 1, 4a, 6-tetramethylnapthalene
7.398	2.95	n-hexadecanoic acid	33.54	1.16	Phenol, 2,2¢-methylenebis [6-(1,1-dimethylethyl)-4-methyl
8.068	0.96	9,12,15- Octadecatrienoic acid	40.27	3.15	8-Morpholino-4-cyclooctene-1-one oxime
8.260	4.88	9,12,-Octadecadienoic acid	43.77	25.76	Unknown
8.369	3.12	Octadecadienoic acid			
9.239	1.47	Eicosanic acid			
10.410	0.92	Docosane			
10.982	3.7	1-Pentadecane			
11.874	12.25	Hexacosane			
12.041	1.96	Tetracosane			
12.267	0.96	1-iodohexadecane			
12.735	1.83	Hentriacontane			
12.735	1.83	1-Docosene			
12.987	1.55	Alpha-Tocopherol			
13.639	2.31	Camposterol			
13.639	2.31	5-Cholesten-3β-ol-7-one			
13.781	1.59	Stigmasterol			
13.781	1.59	Cholesta-6,22,24-triene,4,4-dimethyl			

GC-MS: gas chromatography–mass spectrometry

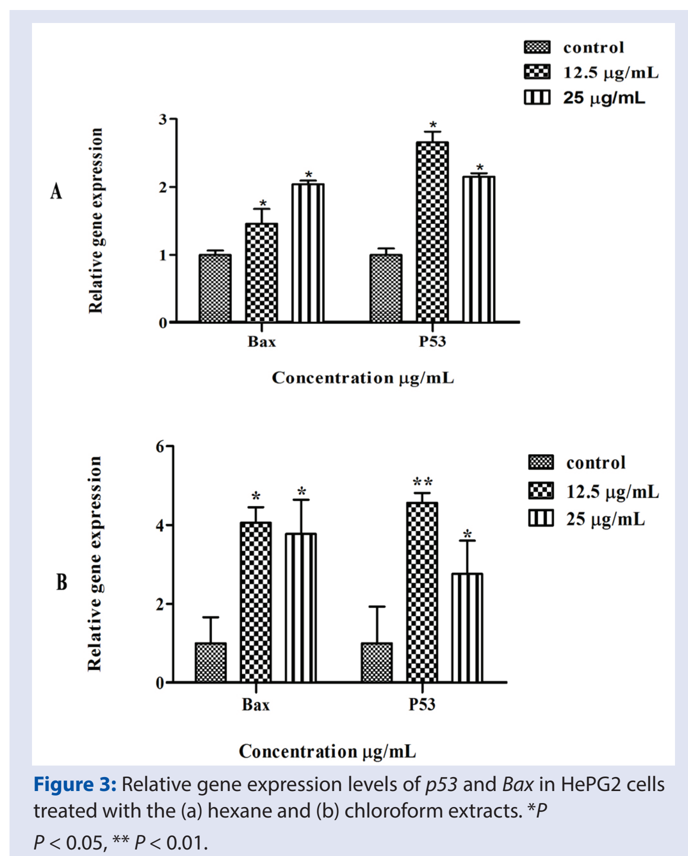


Figure 3: Relative gene expression levels of *p53* and *Bax* in HePG2 cells treated with the (a) hexane and (b) chloroform extracts. *P $P < 0.05$, ** $P < 0.01$.

hepatocellular carcinoma.[17] Results of the present study demonstrated that the hexane and chloroform extracts of *S. hydrophyllacea* leaves exert a strong dose-dependent and time-dependent cytotoxicity on HepG2 cells as assessed by MTT and SRB assays, whereas ethyl acetate and methanol extracts had no significant cytotoxicity. Apoptosis is an essential aspect of the development and health of a multicellular organism. During apoptosis, cell death occurs in a well-controlled and regulated manner.[18] Investigations with many medicinal and other plants have demonstrated that apoptosis is a major mechanism through which they mediate anticancer effects. Results obtained in the present study also confirm that apoptosis could indeed to be a major mechanism through which the hexane and chloroform extracts mediate their antihepatocarcinogenic effect. AO/EB staining of HepG2 cells revealed that morphological changes related to apoptosis occurs in a dose-dependent manner, although a considerable smearing pattern of DNA was observed only in the HepG2 cells treated with the highest dose of chloroform extract.

Two major pathways (extrinsic and intrinsic) are involved in the regulation of apoptosis. Both these pathways converge to a common pathway by specific proteases called "caspases," a family of enzymes that act as death effector molecules in various forms of cell death.[19] Two classes of caspases are generally involved in the regulation and execution of apoptosis: the initiator caspases, which include caspases 2, 8, 9, and 10 and the effector caspases, which include caspases 3, 6, and 7.[20] Results of the present study demonstrate that the hexane and chloroform extracts of *S. hydrophyllacea* activated both caspase-3 and caspase-9 in HepG2 cells in a time-dependent and dose-dependent manner.

Besides, caspases there are a number of genes involved in mediating apoptosis. Bcl-2 family of proteins such as pro-apoptotic Bax and anti-apoptotic Bcl-2 are mainly involved in the regulation of intrinsic pathway of apoptosis.[21] Pro-apoptotic Bax is involved in the regulation of apoptosis via activation of initiator caspases. The *p53* gene is a tumor suppressor gene and it initiates apoptosis by activating expression of pro-apoptotic Bcl-2 family members such as Bax. Another anti-apoptotic protein, Survivin, is also believed to be regulated by *p53*[22] In the present study, a dose-dependent significant upregulation of pro-apoptotic

Bax and p53 were observed in both hexane and chloroform extract-treated HepG2 cells at 24 h after incubation, suggesting activation of apoptosis via a p53-related mechanism. However, p53 expression was slightly reduced at high doses of chloroform and hexane extracts despite enhanced apoptosis. Reasons for this discrepancy are not clear. It is possible that high doses of chloroform and hexane extracts activate other downstream apoptotic molecules such as apoptosis inducing factor.[23] Presence of fragmented DNA after exposure to chloroform extract results from caspase activity; AO/EB staining and gene expression analysis suggest that chloroform extract mediate apoptosis via caspase and p53-dependent pathway, which finally leads to DNA fragmentation. Absence of DNA fragmentation in hexane extract-treated HepG2 cells results from caspase activity; AO/EB staining and gene expression analysis suggest that hexane extract also mediate apoptosis via caspase and p53-dependent pathway without the activation of endonuclease G, which is necessary for apoptotic DNA fragmentation.[24]

Use of medicinal plants with high amount of antioxidant compounds has been suggested as an effective therapeutic method for hepatic damages.[25] Phenolic compounds are known to be potential antioxidants and there is a close correlation between the content of phenolic compounds and antioxidant activity in plant extracts.[26] Flavonoids, which are naturally available in plants, are believed to mediate positive effects on human health.[27] Research on flavonoid derivatives has proved that they contain anticancer, anti-inflammatory, antibacterial, and anti-allergic activity. Flavonoids have also been proven to be highly effective scavengers of most oxidizing molecules.[28] In the present study, ethyl acetate extract of *S. hydrophyllacea* leaves showed the highest total phenolic content followed by chloroform, methanol, and hexane extracts. Methanol extract contained the highest flavonoid content. Interestingly, both the ethyl acetate extract having the highest total phenolic content and the methanol extract having the highest flavonoid content were not cytotoxic. Furthermore, the free radical scavenging activity was low in the ethyl acetate extract, whereas the methanolic extract had the highest free radical scavenging activity. The second highest free radical scavenging activity was seen in the cytotoxic chloroform extract, which had the lowest level of flavonoids and a moderate level of total phenolic content. Both the DPPH and ABTS assays are widely used to evaluate free radical scavenging activities of plant extracts. 2,2-DPPH and ABTS are two free radicals used in these assays, respectively. Our results suggest that the flavonoids in the methanol extract and phenolic content in the chloroform extract are likely to be more potent mediators of free radical scavenging activity than similar compounds in the other extracts tested.

GC-MS analysis of the hexane and chloroform extracts of *S. hydrophyllacea* leaves have shown that the hexane extract is rich in sterols and fatty acids such as B-Sitosterol, octadecadienoic acid, and lupeol, whereas the chloroform extract contain decahydro-8a-ethyl-1,1,4a,6-tetramethylnapthalene, phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-8-morpholino-4-cyclooctene-1-one oxime, and an unknown compound. B-Sitosterol, octadecadienoic acid, and lupeol found in the hexane extract have been reported to be cytotoxic, and apoptosis inducing in other cancer cell lines include human gastric (SGC-7901), hepatocellular carcinoma (BEL-7402), and leukemia (HL-60) tumor cells.[29,30] Further studies are needed to isolate the active antihepatocarcinogenic and antioxidant compounds from the active extracts of *S. hydrophyllacea*

CONCLUSION

Results of the present study demonstrated the presence of anticancer compounds in mangrove plant *S. hydrophyllacea*. Of the four solvent extracts, hexane and chloroform extracts showed greater cytotoxicity to HepG2 cells. Furthermore, these two extracts exert apoptotic and anti-oxidant effects. Cytotoxic and apoptosis-related studies of hexane and chloroform extracts allow us to conclude that these two extracts are good source for isolation of possible anticarcinogenic compounds.

Financial support and sponsorship

International Foundation for Science (IFS) Sweden under grant F/5377-1 is gratefully acknowledged.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Raza A, Sood GK. Hepatocellular carcinoma review. Current treatment and evidence-based medicine. World J Gastroenterol 2014;20:4115-27.
2. Poon RT, Fan ST, Tsang FH, Wong J. Locoregional therapies for hepatocellular carcinoma: A critical review from the surgeon's perspective. Ann Surg 2012;235:466-86.
3. Maorl Y, Malnick S. injury induced by anticancer chemotherapy and radiation therapy. Int J Hepatol 2013;id. 815105: 1-8.
4. Wang H, OoKhor T, Shu L, Su Z, Fuentes F, Lee JH. *et al.* Plants against cancer: A review on natural phytochemicals in preventing and treating cancers and their drug ability. Anticancer Agents Med Chem 2012;12:1281-305.
5. Mathew N, Misra-Bhattacharya S, Perumal V, Muthuswamy K. Antifilarial lead molecules isolated from *Trachyspermumammi.* Molecules 2008;13:2156-68.
6. Jayanta KP, Alok DM, Sakti KR, Nabin KD, Hrudayanath T. Screening of antioxidant and antifilarial activity of *Excoecariaagallocha. L.* Int J Inter Biol 2009;7:9-15.
7. Zeng YB, Mei WL, Zhao YX, Dai HF. Cytotoxic components from mangrove plant *Scyphiphora hydrophyllacea.* J Trop Subtrop Bot 2007;15:249-52.
8. Feng CL, Gong MF, Zeng YB, Dai HF, Mei WL. Scyphiphin C: a new iridoid from *Scyphiphora hydrophyllacea.* Molecules 2010;15:2473-7.
9. Spanos GA, Wrolstad RE. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. J Agr Food Chem 1990;38:1565-71.
10. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem 2005;91:571-7.
11. Mothana RA, Lindequist U, Grunert R, Bednarski PJ. Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from island Soqotra. BMC Complement Altern Med 2009;9:7.
12. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231-7.
13. Samarakoon SR, Thabrew I, Galhena PB, De Silva D, Tennekoon KH. A comparison of the cytotoxic potential of standardized aqueous and ethanolic extracts of a polyherbal mixture comprised of *Nigella sativa* (seeds), *Hemidesmusindicus* (roots) and *Smilax glabra* (rhizome). Pharmacognosy Res 2010;2:335-42.
14. Samarakoon SR, Thabrew I, Galhena BP, Tennekoon KH. Modulation of apoptosis in human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction *of Nigella sativa* seeds, *Hemidesmusindicus* roots and *Smilax glabra* rhizomes with anti- hepatocarcinogenic effects. BMC Complement Alternate Med 2012;12:25.
15. Samarakoon SR, Thabrew I, Galhena BP, Tennekoon KH. Modulation of apoptosis in human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction *of Nigella sativa* seeds, *Hemidesmusindicus* roots and *Smilax glabra* rhizomes with anti- hepatocarcinogenic effects. BMC Complement Alternate Med 2012;12:25.
16. Kennethand LJ, Thomas SD, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001;25:402-8.
17. Aggarwal BB, Prasad S, Reuter S, Kannappan R, Yadev VR, Park B. Identification of novel anti-inflammatory agents from ayurvedic medicine for prevention of chronic diseases. Curr Drug Targets 2011;12:1595-1653.
18. Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol 2007;35:495-516.
19. Kelly GL, Strasser A. The essential role of evasion from cell death in cancer. Adv Cancer Res 2011;111:39-96.
20. Johnson CR, Jarvis WD. Caspase-9 regulation: An update. Apoptosis 2004;9:423-7.

21. Findley HW, Gu L, Yeager AM, Zhou M. Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. Blood 1997;89:2986-93.
22. Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S. *et al*. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613-22.
23. Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 2003;22:4385-99.
24. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 2001;412:95-9.
25. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilisleptophylla L*. BMC Complement Altern Med 2012;12:221.
26. Vamanu E, Nita S. Antioxidant capacity and the correlation with major phenolic compounds, anthocyanin, and tocopherol content in various extracts from the wild edible *Boletus edulis* mushroom. BioMed Res Int 2013;id.313905:1-11.
27. Pandey K, Rizv SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2009;2:270-8.
28. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilisleptophylla* L. BMC Complement Altern Med 2012;12:221.
29. Gallo MBC, Sarachine MJ. Biological activities of lupeol. Int J Biomed Pharm Sci Globle Science Book 2009;3:46-66.
30. Saeidnia S, Manayi A, Gohari AR, Abdollahi M, The story of beta-sitosterol—A review. Eur J Med Plants 2014;4:590-9.