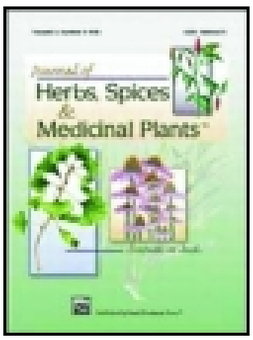

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N.R. Malge[a], A.M.P.W. Bandara[a], W.L.R. Keerthirathna[a], D.M.I. Dissanayake[a], Patharage Kamal Perera[b], Chamindri Witharana[c], and L.D.C. Peiris[a]

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ABSTRACT

The antioxidant, cytotoxic, and anti-cancer potential of aqueous root and rhizome extract (ARRE) of *Smilax zeylanica* was investigated against human liver carcinoma cells. The phytochemical composition was evaluated by standard methods, and confirmed by thin-layer chromatography. Free radical scavenging activity was determined by DPPH assay, cytotoxic activity by brine shrimp lethality assay, and anti-cancer potential was investigated using liver carcinoma HepG2 cells. Phytochemical analysis revealed the presence of saponins, flavonoids, proanthocyanins, tannins, and polyphenols. The total phenolic and flavonoid content varied from 0.28 to 5.26 mg gallic acid equivalent 100 mg^{-1} and 0.25–50.26 quercetin equivalent 100 mg^{-1}, respectively. There was a linear relationship between the free radical scavenging activity and phytochemical concentrations with an IC_{50} value of 108 ± 1.2 µg mL^{-1} compared to the standard (IC_{50}: 2.59 ± 0.02 µg mL^{-1}). The brine shrimp lethality assay showed strong cell killing ability (IC_{50}: 84 ± 0.7 µg mL^{-1}) and the extract induced apoptosis of HepG2 cells (77 ± 1.52%) at 300 µg mL^{-1}, comparable to the positive control (camptothecin).

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KEYWORDS
aqueous extract; hepato carcinoma; cell killing; S. zeylanica

Introduction

Hepatocellular carcinoma incidences ranked fifth among the most prevalent cancers globally, with ~ 60,000 annual deaths. Chronic liver infections, obesity, and alcohol abuse can result in oxidative stress and lipid peroxidation, resulting in reactive oxygen species.[1] Oxidative stress plays a crucial role in hepatocarcinogenesis. Genetic factors may also contribute to liver cancer by inheriting susceptible genes from their parents.[2] Current treatments by surgery, or chemotherapy for liver carcinoma patients are accompanied by potential short-term and long-term side effects including hair-loss, reproductive effects

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on neural disorders. Plant-based remedies are popular among Ayurveda and other modes of traditional medicine due to minimal side effects.[3] Focus on plants with high polyphenolic content has increased due to their documented antimicrobial, chemoprotective potential,[4] and selective cell-killing ability.[5]

Plants are rich in secondary metabolites that demonstrate therapeutic properties acting individually or in combination.[6–8] Advanced chemical studies have demonstrated that secondary metabolites rich in antioxidant and cytoprotective properties possess potential chemopreventive and therapeutic activities against malignant tumors and inflammatory disorders.[9–12] Plant secondary metabolites interact with free radicals to neutralize their adverse effects by converting them to nontoxic compounds.[13] Phenolic compounds have provided a strong defense against cellular damages caused by free-radical induced oxidative stress.[5]

About 60% to 80% of the world's population still relies on traditional medicine against various diseases.[14] *Smilax zeylanica* (Similaceace) is frequently prescribed by traditional Sri Lankan practitioners against a variety of ailments such as arthritis and skin diseases and the roots and rhizomes are widely utilized against cancer. The plant is a perennial climber native to Sri Lanka but distributed throughout the tropics.[15] It is commonly known as *Kabarassa, Kabarossa, Kabalossa* in Sinhala, and *Ayadi* in Tamil. The antioxidant activity of methanol extracts of roots, stems, and aerial parts of *S. zeylanica* has been documented.[16–18] Since in traditional medicine aqueous extracts are used, this study examined the aqueous extract of root and rhizomes from *S. zeylanica* for potential antioxidant, cytotoxic, and anti-cancer activities.

Materials and Methods

Chemicals

All chemicals were of analytical grade and obtained from Sigma Aldrich Co.; St. Louis; USA unless otherwise stated.

Collection and Identification of Plant Materials

Fresh roots and rhizomes of *S. zeylanica* were collected from mature, healthy plants from Galle, Sri Lanka (6.0367°N; 80.217°E) from March to September. The plant was authenticated by the Faculty of Indigenous Medicine, University of Colombo and a voucher specimen was deposited in the Department of Pharmacology. The roots and rhizomes were washed thoroughly, cut into small pieces, shade-dried for six weeks and ground to a powder using a domestic grinder.

Preparation of Root and Rhizome Extract

The powdered root and rhizome (25 g) were mixed in 150 mL distilled water and subjected to sonication at 25°C for three 90 sec. Obtained aqueous root and rhizome extract (ARRE) was filtered through Whatman filter paper no. 1, freeze-dried, and stored at – 20°C until further use.

Phytochemical Analysis

Qualitative phytochemical screening of the extract was conducted as described.[17] The aqueous root and rhizome extracts (ARRE) were screened for alkaloids, unsaturated sterols, triterpenes, saponins, flavonoids, proanthocyanidins, anthraquinones, tannins, polyphenols, and cyanogenic glycosides. The presence of flavonoids in the extract was confirmed using thin-layer chromatography (TLC) on pre-coated Kieselgel 60 F_{254} solvent systems using ethyl acetate: formic acid: dichloromethane: methanol (6.8: 0.2: 2.8: 0.2). The development of spots was observed under UV light before and after spraying the natural product reagent at 254 nm and 365 nm, respectively.

Total Phenolic and Flavonoid Content

The total phenolic content was determined using the Folin-Ciocalteu colorimetry.[18] Briefly, different concentrations (0.25, 0.5, 1 mg mL^{-1}) of ARRE of *S. zeylanica* were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with 2% Na_2CO_3. The concentrations were selected based on a previous study.[16] The absorbance of the resulting blue color was read at 760 nm (UV visible spectrophotometer; Labomed, Inc. USA) against a blank. The results were expressed as mg gallic acid equivalents (GAE) g^{-1} of the extract.

To determine the total flavonoid content, about 500 µL of different concentrations of ARRE (0.25, 0.5, 1 mg mL^{-1}) of *S. zeylanica* were incubated with 2 mL of distilled water[19] for 5 min. Subsequently, 5% $NaNO_3$ 10% $AlCl_3$, and 1 M NaOH was added, incubated at room temperature and the final volume of the test tube was made up to 5 ml with distilled water. The resulting pink color was measured at 510 nm against a blank reagent. Total flavonoid content was calculated from the calibration curve plotted using standard quercetin (20, 40, 60, 80, 100 µg mL^{-1}) and expressed as mg of quercetin equivalent (QE) g^{-1} of dried plant material.

DPPH Free Radical Scavenging Ability

The DPPH (1;1-diphenyl-2-picrylhydrazyl) free radicals[20] potential of the extract was evaluated using different concentrations (1–10^{-8} mg mL^{-1}) of ARRE and the standard ascorbic acid. Each concentration (1.5 mL) was added to three separate test tubes and mixed with an equal quantity of DPPH solution. Reaction mixtures were allowed to stand at room temperature for 30 min in the dark, and the discoloration was measured at 517 nm using a UV-Visible spectrophotometer. The DPPH solution mixed with 1.5 mL of methanol in the absence of samples/standard was used as control, and methanol as blank. The DPPH scavenging percentage was plotted against the tested concentrations to obtain the IC_{50} values for the extraction and the positive control.

Brian Shrimp Lethality Assay

Brine shrimp lethality bioassay was carried out to test the cytotoxicity of ARRE of *S. zeylanica* as described[21] with some modifications. Brine shrimp (*Artemia salina*) cysts were purchased (Fish paradise aquarium Nugegoda; Sri Lanka) and hatched in a conical flask filled with artificial seawater (32 ppm) under constant aeration for 48 h and artificial illumination (60 W). Concentrations ranging from 8 to 666 μg mL^{-1} of ARRE was prepared by dissolving in aerated artificial seawater (32 ppm). Artificial seawater (32 ppm) was used as the negative control, while a standard potassium dichromate solution was used as positive control. Active nauplii larvae (age: 48 h; n = 50) were introduced to the test compounds and kept under the light for 24 h at room temperature. The survivors were counted under a dissecting microscope after incubation. Death percentage was calculated to determine the lethal concentration (LC_{50}).

$$\text{Percentagedeath}(\%) = [(\text{Totalnauplii} - \text{livenauplii})/\text{totalnauplii}] \times 100.$$

Cell Line and Culture

An anti-proliferative activity using HepG2 cell line (ATCC) was conducted at the Department of Biochemistry, University of Colombo. Cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Growth was maintained at 37°C in a humidified incubator containing 5% CO_2.

Anti-proliferative Assay

Anti-proliferative activity was conducted using 3-(4, 5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as described.[22] Briefly; the

HepG2 was seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µl EMEM and incubated 48 h with different concentration of ARRE (10–750 µg mL^{-1}) at 37°C in a 5% CO_2 incubator. After incubation, 20 µL of MTT reagent was added, and plates were incubated further for 4 h in the dark. The apoptosis activity of the extract was determined using an ELISA reader at 590 nm. The percentage inhibition of cell growth was determined and the anti-proliferative effect of ARRE was expressed as EC_{50} value. The media without the sample served as negative control, and 5 µg mL^{-1} camptothecin as positive control.

Statistical Analysis

Each experiment was conducted in triplicates and the data presented as Mean ±SEM. The data were analyzed by Minitab16 2.4.0 (Minitab Inc., State College, PA, USA) by one-way ANOVA followed by Duncan's tests ($P < .05$). IC_{50} values were calculated by Microsoft Office Excel 2010.

Results

Phytochemical Analysis

Qualitative analysis of phytochemicals in crude extracts indicated the presence saponin, polyphenols, proanthocyanin, flavonoids, and tannins. Since the presence of flavonoids and proanthocyanidins can result in false-positive presence under acidic conditions of the test carried out for flavonoids, the presence of flavonoids was confirmed by TLC, which resulted in an orange-yellow color spot under UV-364 nm wavelength after spraying the NPR reagent (Fig. 1).

Quantification of Total Phenol and Flavonoid Contents

The phenolic content varied between 0.61 and 5.06 mg GAE g^{-1} extract, and the flavonoids ranged from 5.29 to 50.26 QE g^{-1} extract. The highest phenolic and flavonoid contents were noted at 1 mg mL^{-1} of ARRE of *S. zeylanica*. The extract was ten times richer in flavonoids than phenolics (Table 1).

In Vitro Antioxidant Activity

The investigated antioxidant potential of the extract was concentration-dependent ($R^2 = 0.986$) with DPPH free radical scavenging activity (Fig. 2). The IC_{50} of the ARRE was 108.00 ± 1.2 µg mL^{-1} suggesting a moderate antioxidant potential. IC_{50} for positive control (ascorbic acid) was 2.59 ± 0.02 µg mL^{-1}.

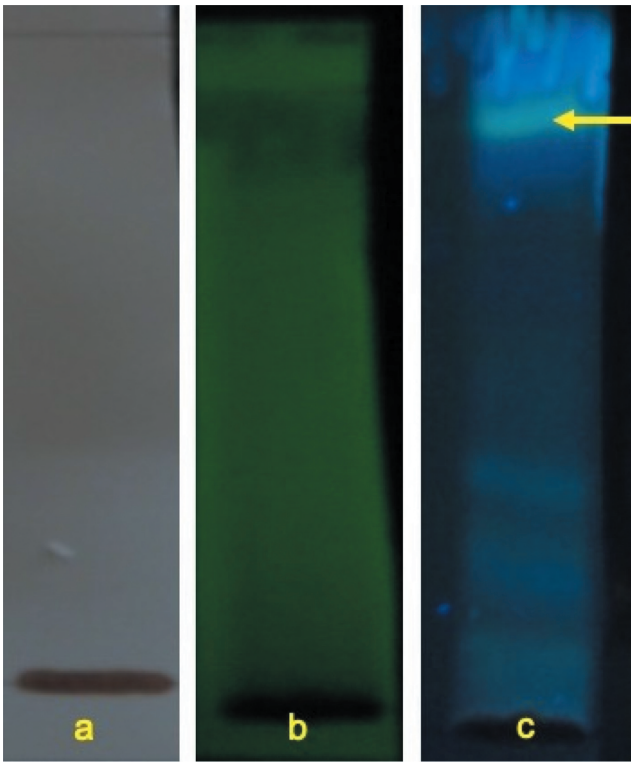

Figure 1. Thin layer chromatography observed under UV light (a): before spraying natural product visualizing reagent (NPR); (b): after spraying NPR; observed under UV-254 mm; and (c): after sparing NPR and observed under UV-364 mm. A yellow spot appeared at UV-364 (indicated by the yellow-colored arrow) confirmed the presence of flavonoid.

Table 1. Total phenolic and flavonoid contents of aqueous extract of *Similaz zeylanica* roots and rhizomes.

Concertation (mg mL^{-1})	Total phenolic content (mg GAE g^{-1} extract)	Total flavonoïde content (mg QE g^{-1} extract)
0.25	0.61 ± 0.06	5.29 ± 0.15
0.5	1.85 ± 0.24	11.91 ± 1.03
1	5.06 ± 0.41	50.26 ± 1.26

Values are mean ± SEM; n = 3. GAE: gallic acid equivalent, QE: quercetin equivalent.

Brine Shrimp Lethality Assay

The mortality rate of brine shrimp displayed by ARRE of *S. zeylanica* increased with increasing concentrations (Fig. 3). The negative control (artificial seawater: 32 ppm) did not exert toxic effects on brine shrimps. The LC_{50} value obtained for the root extract (LC_{50}: 83 ± 0.7 µg mL^{-1}) was lower than in the positive control (LC_{50}: 35.6 ± 0.2 µg mL^{-1}), indicating intense cell-killing activity of the aqueous extract. Plant extracts exhibiting LC_{50} values <100 µg mL^{-1} exhibit strong cytotoxic potentials and hence *S. zeylanica* may be considered as a robust cytotoxic agent.[23]

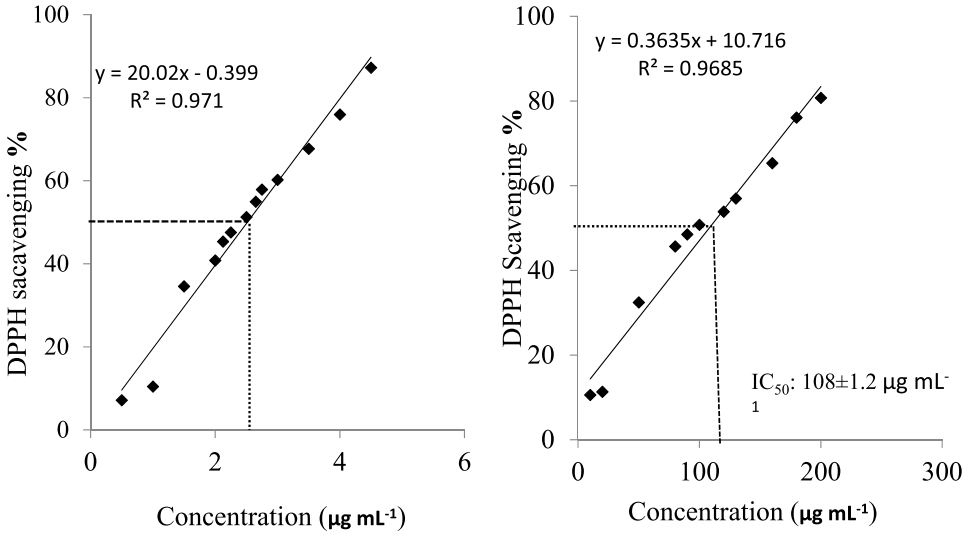

Figure 2. DPPH scavenging percentage of (a); aqueous extracts of *Smilax zeylanica* roots and rhizomes and (b): positive control (acarbose). Data are mean ± SEM (n = 3).

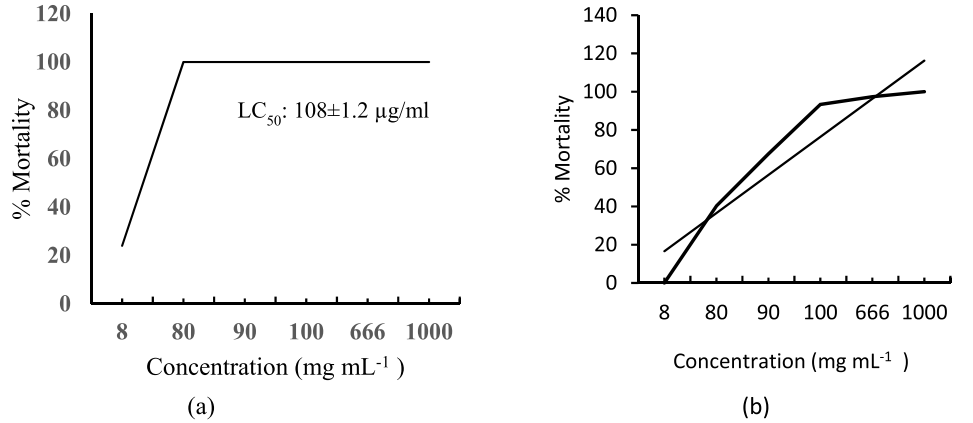

Figure 3. Percentage mortality of *Artemia salina* larvae induced by (a): positive control (potassium dichromate) and (b): aqueous extract of *Smilax zeylanica* roots and rhizomes. Data are mean ± SEM (n = 50).

Anti-cancer Potential

The root extract of *S. zeylanica* was investigated for the anti-cancer property against human liver carcinoma cells (HepG2) using MTT assay upon 48 h exposure. The root and rhizome extract of *S. zeylanica* exhibited potent anti-cancer activity with an IC_{50} value of 49.9 ± 0.1 µg mL^{-1} (Fig. 4). The cell proliferation was inhibited with increasing concentration of ARRE. At the lowest dose, the cell growth was inhibited by 14.5%. The highest concentration

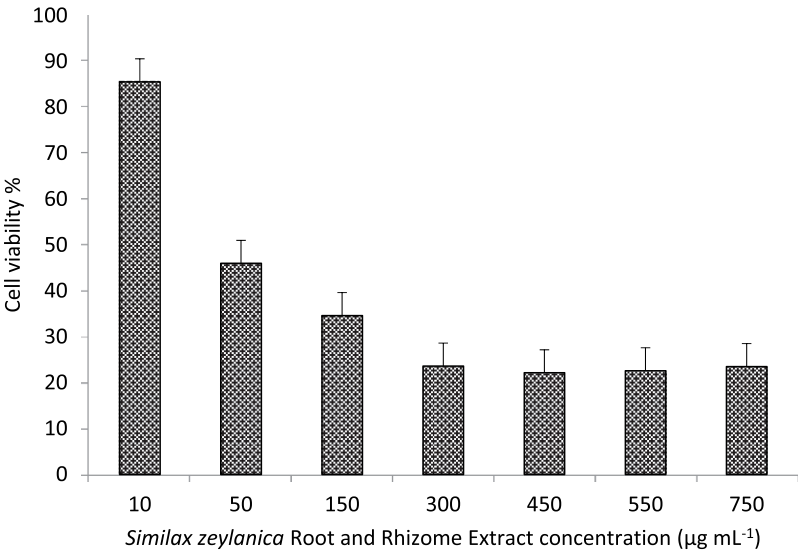

Figure 4. Cell viability percentage of the HepG2 cell line as determined by MTT assay, after 48 h treatment with aqueous extract of roots and rhizomes from *Similax zeylanica*. Values are mean ± SEM (n = 3).

of ARRE (300 µg mL^{-1}) exhibited the highest inhibition percentage (77.05 ± 1.53%), which was similar to positive control (76.05 ± 1.5%). The cell viability remained stable at concentrations higher than 300 µg mL^{-1}.

Discussion

The roots and rhizomes of *S. zeylanica* are commonly prescribed by traditional Ayurvedic practitioners to combat cancer. Increased production of reactive oxygen species can be observed due to lipid peroxidation, which increased when a person has a disease that may damage membrane lipids, nucleic acids, carbohydrates, and proteins.[14] Plants are potential sources of natural antioxidant compounds. Their protective effect has been attributed to components such as phenolic acids, vitamin C and carotenoids. The total phenolics in the ARRE of *S. zeylanica* was 5.16 mg GAE g^{-1} extract at 1 mg mL^{-1}, that results in the antioxidant activity observed in the extract. These compounds are known to possess redox properties and, therefore, can act as reducing agents and hydrogen donors. In the present study, antioxidant activity was evaluated using DPPH activity, which evaluated the potential of the ARRE of *S. zeylanica* to reduce DPPH free radicals *via* hydrogen donating ability. The ARRE of *S. zeylanica* produced a dose-dependent protective effect against reducing free-radicals generated by DPPH, and the detected free radical scavenging ability may be attributed to the presence of the phenolics and flavonoids.[24] In contrast to the documented literature,[15] the

antioxidant capacity of the aqueous extract of *S. zeylanica* exhibited in the present study was more potent (IC_{50}: 125 ± 0.13 μg mL^{-1}), which may be due to the geographical differences of the study locations. However, the antioxidant activity exerted by the ARRE of *S. zeylanica* was more potent than that reported for the aqueous extract of *S. spinosa*.[25]

Brine shrimp lethality assay is one of the easiest, reliable, and cost-effective assays that correlates with the cytotoxic and anti-proliferative properties.[26] Previous studies documented a positive relationship between the brine shrimp cytotoxic assay and human tumors.[27] The aqueous extract exerted 50% mortality of brine shrimp at 84 μg mL^{-1}. According to standard brine shrimp lethality assay, plant extracts exhibiting LC_{50} values < 100 μg mL^{-1} are considered "highly cytotoxic".[23] As per this standard, the ARRE of *S. zeylanica* with an LC_{50} value of 84 ± 0.7 μg mL^{-1} may be considered as having potent cell-killing ability, further confirmed by MTT assay carried out against HepG2 cells.

The aqueous extract exhibited potent anti-cancer activity *in vitro* against HepG*2* carcinoma cells at 300 μg mL^{-1}. The anti-cancer activity remained constant at doses higher than 300 μg mL^{-1}. The IC_{50} value is the dose at which 50% cell growth was inhibited, suggesting intense inhibitory activity. The IC_{50} value of the *S. zeylanica* ARRE was 49 μg mL^{-1}, which implies potent anti-cancer activity. The standard camptothecin resulted in 76% inhibition of tested liver carcinoma cells at a dose level equivalent to 900 μg mL^{-1}. On the contrary, the *S. zeylanica* extract inhibited 77% cell growth at 300 μg mL^{-1}. Phenols and flavonoids are effective anti-cancer agents, and they act by inducing cell cycle arrest, apoptosis, and anti-angiogenic activities.[28,29] Thus, high flavonoid and phenolic contents in the *S. zeylanica* ARRE may be responsible for the cell-killing ability exhibited. Quantitative and qualitative phytochemical screening and TLC tests confirmed the presence of phenolic compounds in extract. The results suggest that the anti-cancer efficacy of the *S. zeylanica* ARRE is greater than the standard used in this study. Hence, the aqueous extract may be used to develop anti-cancer drug leads. The anti-cancer activity of the AERR was more potent than the recorded cytotoxicity of *S. spinose* ethyl acetate extract against HepG2 cells.[25] The aqueous extract is rich in flavonoids, polyphenols, and proanthocyanin, which are known to possess anti-proliferative activity, especially against liver cancer cells.[30,31]

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Competing Of Interest

The authors declare no competing interests.

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