Cytotoxicity against Human Hepatocellular Carcinoma (HepG2) Cells and Anti-Oxidant Activity of Selected Endemic or Medicinal Plants in Sri Lanka

Jeyaraj Thusyanthan, Nimesha Sulochani Wickramaratne, Kanishka Sithira Senathilake, Umapriyatharshini Rajagopalan, Kamani Hemamala Tennekoon, Ira Thabrew, and Sameera Ranganath Samarakoon

Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo, No. 90, Cumaratunga Munidasa Mawatha, Colombo-3, Sri Lanka

Correspondence should be addressed to Sameera Ranganath Samarakoon; sam@ibmbb.cmb.ac.lk

Received 24 November 2021; Revised 20 January 2022; Accepted 24 February 2022; Published 30 March 2022

1. Introduction

Liver cancer is one of the cancers with a high mortality rate worldwide, and it is challenging to cure. The highest incidence of liver cancer is in Asia and Africa. More than 75% of liver cancer incidents were reported from Asian countries. Epidemics of liver cancers are increased around the world including India and the USA [1]. A significant number of patients were also reported in Sri Lanka in past years. Based on the National Cancer registry published in 2020, there were 229 males (2.1%) and 92 females (0.8%) reported for HCC out of 11,773 cancer patients in 2012 [2]. Hepato-cellular carcinoma (HCC) is a primary cancer of the liver that is predominant in developing countries [3]. HCC is occurred by the presence of hepatocellular damage through reactive oxygen species and the generation of chronic inflammation related to hepatocarcinogenesis [4]. The human body reacts in various ways to counteract hepatocarcinogenesis. One such way is apoptosis or programmed cell death that maintains homeostasis between cell death and cell
proliferation. Nuclear fragmentation and nuclear condensation are major morphological changes that occur during apoptosis. Cells that acquire morphological changes are phagocytized by macrophages. Apoptosis is mediated via either extrinsic (death receptor), intrinsic mitochondrial pathway, or intrinsic endoplasmic reticulum pathway. Therefore, selective induction of apoptosis of cancer cells is one of the targets for the treatment of cancer [5].

Traditional medical practitioners in Sri Lanka use plants and plant-based formulations to treat cancers [6]. Plants are a major source of a variety of secondary metabolites including phenolic (e.g., phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen-containing compounds (e.g., alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and other endogenous metabolites [7–13]. Flavonoid and phenolic acids are important classes of secondary metabolites capable of scavenging free superoxide radicals, promoting anti-aging, and reducing the risk of cancer [14–16] and improve plant aroma, coloration, and flavor [17]. Phenolic compounds act as protective agents against invading plant pathogens [18–21]. Phenolic and flavonoids are reported to exhibit anti-ulcerative, anti-inflammatory, anti-oxidant, cytotoxic, anti-cancer, and anti-depressant activities [20]. Intake of phenolic compounds reduces the risk of degenerative diseases accompanied with oxidative stress including cardiovascular disease, diabetes, and obesity [22]. Anti-oxidant activity is a fundamental property of most of the bioactive compounds with anti-carcinogenic and anti-aging properties [23–25].

Different treatment modalities including radiation therapy, chemotherapy, and surgery are currently used in the treatment of liver cancer. However, a successful anti-liver cancer drug with no or minimum toxic effects is unavailable to date. Sri Lanka having rich biodiversity possesses plants that have not yet been systematically investigated for both their chemical composition and bioactivities. Therefore, the current study was undertaken to screen and identify potent Sri Lankan plant sources that can provide novel and effective anti-liver-cancer compounds as well as to characterize their anti-oxidant activities.

2. Materials and Methods

2.1. Chemicals and Reagents. Human hepatocellular carcinoma HepG2 (HB 8065™) and human normal liver epithelial cell line THLE-3 (CRL-11233™), Dulbecco’s Modified Eagle’s Medium (DMEM-Cat. No. 30-2002), fetal bovine serum (FBS-Cat. No. 30-2020), trypsin-EDTA (Cat. No. 30-2300), were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Bronchial Epithelial Cell Growth Medium BulletKit™ (BEGM- CC-4175) and BEGM (ATCC; St. Louis, MO, USA).

2.2. Cell Lines and Maintenance of Cell Culture. The human hepatocellular carcinoma cell line (HepG2) and the normal liver epithelial cell line (THLE-3) were maintained according to ATCC guidelines. HepG2 and THLE-3 cells were cultured in DMEM and BEGM, respectively with 0.1% streptomycin/penicillin and 10% fetal bovine serum. Both cells were incubated in a humified incubator at 37°C under an atmosphere containing 5% CO2. The cells were subcultured at preconfluent densities using 0.25% trypsin-EDTA.

2.3. Collection and Authentication of Plant Material. Fresh and healthy leaves and bark of seven selected endemic and medicinal plants were collected from Pitigala, Kaluthara district, the western province of Sri Lanka. Plants were identified by the Botanists at the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. Voucher specimens were deposited, and the details are given in Table 1.

2.4. Preparation of Plant Extracts. Plant leaves and barks were air-dried and ground into fine powder. Ground powder (5 g each) was sequentially extracted in hexane, dichloromethane, ethyl acetate, and methanol by sonication. Extracts were air-dried and ground into fine powder. Gound powder (5 g each) was sequentially extracted in hexane, dichloromethane, ethyl acetate, and methanol by sonication. The extracts were then filtered and dried in a rotary evaporator at room temperature. Stock solutions were prepared by dissolving dried extracts in appropriate amount of dimethyl sulfoxide (DMSO). The dilution series of each extract was prepared by adding DMEM containing 10% FBS.

2.5. Cytotoxicity Assay. Sulforhodamine B (SRB) assay was performed to determine cytotoxicity (IC50 value) of plant extracts against HepG2 cells as described by Samarakoon et al. [26] with some modifications. HepG2 cells were seeded into a 96-well plate (5,000 cells/well) with 200 μL of DMEM containing 10% of FBS and incubated at 37°C for 24 h. Cells cultured in vitro were exposed in triplicates to each plant extract (12.5–200 μg/mL) and incubated for 48 h. Thymoquinone (1.5–25 μg/mL) was used as the positive control. Cells were observed under a light microscope after 48 h of incubation, and an SRB assay was performed to determine cytotoxicity. Absorbance was read using Synergy™ HT multimode microplate reader at 540 nm. Percentage of cell viability was calculated, and IC50 values were determined using GraphPad Prism® software (version 6.01). Extracts identified to have potent cytotoxicity (IC50 < 100 μg/mL) on HepG2 cells were also tested on the normal liver epithelial cell line (THLE-3), and cytotoxicity was determined after 48 h of incubation.

2.6. Detection of Morphological Changes by Light Microscopy. HepG2 cells were exposed to different concentrations (12.5–200 μg/mL) of each plant extract for 48 h. After the
exposure period, cells were observed under an inverted light microscope (Olympus CKX41SF, Japan) to detect any morphological changes related to apoptosis.

2.7. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine radical scavenging activity as described by Ediriweera et al. with some modifications [27]. Dilution series (7.825–1,000 μg/mL) of each plant’s leaf and bark extracts were prepared using dimethylsulfoxide (DMSO) in triplicates. Sample (50 μL), DPPH dye reagent (90 μL of 2 mg/mL), and DMSO (60 μL) were added, mixed well, and incubated in the dark for 30 min. Absorbance was measured at 517.0 nm with Synergy™ HT multimode microplate reader. Percentage of inhibition was calculated according to the following equation:

\[
\% \text{ inhibition} = \left( \frac{\text{ABS}_{\text{control}} - \text{ABS}_{\text{sample}}}{\text{ABS}_{\text{control}}} \right) \times 100. \tag{1}
\]

where \( \text{ABS}_{\text{control}} \) = absorbance of control and \( \text{ABS}_{\text{sample}} \) = absorbance of sample. The effective concentration of sample required to scavenge 50% (EC₅₀) was determined by linear regression analysis of the curve plotted between percentage inhibition versus concentration. Ascorbic acid was used as a positive control. Extracts having EC₅₀ < 100 μg/mL were considered as having considerable anti-oxidant activity.

2.8. Ferric Reducing Anti-Oxidant Power (FRAP) Assay. Ferric reducing anti-oxidant power (FRAP) was used to determine the anti-oxidant activity of plant extracts as described by Benzie and Strain [28]. Dilution series (7.825–1,000 μg/mL) was prepared using acetate buffer (pH 3.6). Sample (20 μL) acetate buffer (70 μL) and FeCl₃ solution (60 μL) were mixed to take a preabsorbance reading. Prepared 50 μL of FRAP reagent (acetate buffer:FeCl₃:2,4,6-tripyridyl-s-triazine (TPTZ) as 10:1:1) was added, mixed, and incubated at room temperature for 15 min, and the absorbance was measured at 500 nm using acetate buffer as the blank. Trolox was used as the positive control. Anti-oxidant activity was determined as w/w of Trolox equivalents (TE in mg/g). Extrakts having Trolox equivalents greater than 100 mg/g were considered as having potent anti-oxidant activity by FRAP assay.

2.9. Total Phenolic Content. The total phenolic content of the plant extracts was determined by the Folin–Ciocalteu method described by Ediriweera et al. with some modifications [27]. Dilution series (7.825–1,000 μg/mL) was prepared using distilled water in triplicates. Sample (20 μL) and Folin–Ciocalteu’s reagent (110 μL) were mixed, and the initial absorbance reading was measured at 765 nm using distilled water as the blank. A total of 10% of Na₂CO₃ solution (70 μL) was added, mixed, and incubated for half an hour, and the final absorbance was determined. Gallic acid was used as the positive control. The phenolic content of the test samples was calculated and expressed as w/w dry weight of gallic acid equivalents (GAE in mg/g).

2.10. Total Flavonoid Content. The total flavonoid content of the plant extracts was determined by a modified Dowd method as described by Ediriweera et al. [27]. Dilution series (7.825–1,000 μg/mL) of extracts was prepared in methanol. The initial absorbance was recorded at 415 nm using Synergy™ HT multimode microplate reader. Freshly prepared AlCl₃ solution (100 μL) was added to 100 μL to each dilution and mixed thoroughly. The reaction mixture was mixed well and incubated for 10 minutes at room temperature, and final absorbance reading was taken at 415 nm using methanol as the blank. Quantification was done using a standard curve for quercetin. The results were expressed in quercetin equivalents (QE in mg/g).

2.11. Statistical Analysis. Statistical analysis was performed with GraphPad Prism® software version 6.01 (GraphPad Software Inc., San Diego, CA, USA). The results of anti-oxidant activity, total phenolic content, and total flavonoid content were expressed as mean ± standard deviation of three independent experiments. One-way analysis of variance (one-way ANOVA) was used to compare multiple data. The Pearson correlation coefficient was used to calculate the correlation between two variables, while statistical significance was determined by the two-tailed t-test. It was considered that \( p < 0.05 \) was statistically significant [29].

3. Results and Discussion

Medicinal plants play a major role as therapeutic agents in traditional medicine in developing countries [16]. In the

| Scientific name of the plant | Family name | Endemic/native | Used to treat |
|-----------------------------|-------------|----------------|--------------|
| Allophylus coccineus         | Sapindaceae | Native         | Fracture     |
| Madhuca longiflora          | Sapotaceae  | Native         | Gastritis, bleeding from fresh wounds |
| Adenanthera bicolor         | Fabaceae    | Endemic        | Pruritus, oral ulcer, throat inflammation, stomatitis, wounds |
| Cyclnea peltata             | Menispermaceae | Native     | Fever, dysentery, general pain, swelling, haemorrhoids, cough, asthma, malaria, vomiting, blood disorder |
| Carallia brachiata          | Rhizophoraceae | Native   | Fever, dysentery, general pain, swelling, haemorrhoids, cough, asthma, malaria, vomiting, blood disorder |
| Munronia pinnata            | Meliaceae   | Native         | Native Fracture, bleeding from fresh wounds |
| Schumacheria castaneifolia  | Dilleniacae | Endemic        | Native Urticaria, fraxtigue, snakebite, rheumatism, skin disease |

Table 1: Scientific name, family name, and its usage for treatment/s of selected endemic and medicinal plants.
present study, selected endemic and/or medicinal plants that are used in traditional medicine in Sri Lanka were screened for cytotoxicity. Fifty-six extracts that included hexane, dichloromethane, ethyl acetate, and methanol extracts from the leaves and bark of seven endemic and/or medicinal plants were assessed for anti-cancer activity using HepG2 cells in vitro (Table 2). The normal liver epithelial cell line (THLE-3) was used as the representative of normal human cells for assessing the toxicity of the plant extracts.

Among the extracts tested, dichloromethane extract of Adenanthera bicolor bark; hexane, dichloromethane, ethyl acetate, and methanol extracts of Allophylus cobbe leaves; methanol extract of Munronia pinnata bark; ethyl acetate and methanol extracts of M. pinnata leaves; hexane, dichloromethane, ethyl acetate, and methanol extracts of Schumacheria castaneifolia bark; and hexane, dichloromethane, and ethyl acetate extracts of S. castaneifolia leaves exerted potent cytotoxicity (IC50 < 100 μg/mL) against HepG2 cells (Table 2).

All the extracts of A. cobbe leaves (hexane, dichloromethane, ethyl acetate, and methanol) were highly cytotoxic against HepG2 with low IC50 values (<20.0 μg/mL) and showed less cytotoxicity (150 < IC50 < 300 μg/mL) to THLE-3 cells. Dichloromethane extract of A. cobbe leaves showed the highest cytotoxic effects (IC50 = 6.8 μg/mL) among all the extracts. Thus, the extracts of A. cobbe leaves appear to contain potential cytotoxic compounds that target liver cancer cells. Hexane, dichloromethane, and ethyl acetate extracts of A. cobbe exhibited less anti-oxidant activity with EC50 > 100 μg/mL and TE < 15 mg/g in DPPH and FRAP assays, respectively, along with very low quantity of total phenolics (GAE < 10 mg/g). Methanol extract A. cobbe leaves showed the best anti-oxidant activity (EC50 < 100 μg/mL and TE > 150 mg/g) with significance correlation (p < 0.05). Total phenolic and total flavonoid contents of methanol extract of A. cobbe leaves are comparatively higher than that of other extracts of A. cobbe leaves (80–100 mg/g of GAE and 30–40 mg/g of QE, respectively) with no correlation to anti-oxidant activity (p > 0.05). Previous investigators have reported a lower cytotoxic (IC50) activity for a water extract of A. cobbe leaves (431.10 ± 15.05 μg/mL for DU-145 and 362.08 ± 24.17 μg/mL for PC-3 cell lines at 72 h) though it had a higher total phenolic content (91.96 ± 0.61 mg/g GAE) [30]. The viability of HepG2 cells decreased with increasing concentrations of hexane, dichloromethane, ethyl acetate, and methanol extracts of A. cobbe leaves after 48 h of exposure (Figures 1(a)–1(d)), and the characteristic apoptotic changes including shrunken cells and reduced cell volume (compared to the control) were observed in cells treated with these extracts after 48 h exposure (1A, 1B, 1C, and 2A from Figure 2).

In contrast, none of A. cobbe bark extracts tested exhibited significant cytotoxicity against HepG2 cells at 48 h postincubation (IC50 > 250 μg/mL). Methanol extract of A. cobbe bark extract exhibited better anti-oxidant activity (EC50 < 60 μg/mL and TE > 300 mg/g) with significant correlation (p < 0.05) and contained a considerable quantity of total phenolic (82.43 ± 8.60 mg/g GAE) without significant correlation (p > 0.05). Other extracts excluding methanol extract of A. cobbe bark had low anti-oxidant activity (EC50 > 900 μg/mL and TE < 60 mg/g) along with low content of total phenolics (<20 mg/g GAE) and total flavonoids (<20 mg/g QE).

An endemic plant, A. bicolor leaves exhibited potent cytotoxicity (IC50 < 60 μg/mL) for all four extracts but did not possess potent anti-oxidant activity (EC50 > 200 μg/mL). A steep drop in percentage cell viability was observed in HepG2 cells exposed to increasing concentrations of hexane, dichloromethane, and ethyl acetate extracts of A. bicolor leaves after 48 h (Figures 1(e)–1(g)). The curve was flattened to approximately zero level from the concentration of 50 μg/mL in the above three extracts. Reduced number of cells and constrained cells were visualized through the light microscope (2B, 2C, and 3A of Figure 2). Morphological changes in treated cells indicated that the hexane and dichloromethane extracts of A. bicolor leaves are more cytotoxic to HepG2 cells than the ethyl acetate extract when exposed for 48 h. Methanol extract of A. bicolor leaves exhibited better anti-oxidant activity for FRAP assay (approximately 195 mg/g TE). All the other extracts of A. bicolor leaves exhibited less anti-oxidant activity for FRAP assay (<5 mg/g TE) with a very low amount of total phenolics (<3 mg/g GAE) and total flavonoids (<8 mg/g QE). Results suggest that A. bicolor leaves may contain phenolic compound/s with anti-oxidant activity and cytotoxic activity. Dichloromethane extract of bark exhibited cytotoxicity (IC50 < 50 μg/mL) against HepG2 cells after 48 h exposure. However, it was also toxic to normal hepatocytes (THLE-3) with an IC50 of 69.8 μg/mL. A high percentage of dead cells in the wells (3B of Figure 2) and rapid decrease in percentage cell viability (Figure 1(h)) provide strong evidence for the cytotoxicity of dichloromethane extract of A. bicolor bark to HepG2 cells at 48 h. This extract did not exhibit anti-oxidant activity (EC50 > 300 μg/mL and TE < 5 mg/g) and had low total phenolic (<10 mg/g GAE) and total flavonoid content bark (<10 mg/g QE). Methanol extract of A. bicolor bark had better anti-oxidant activity (>2,000 μg/mL and <40 mg/g TE) with a good quantity of total phenols (>300 mg/g GAE) with significance correlation (p < 0.05); however, it was not cytotoxic (IC50 > 1,000) to HepG2 cells. There was no significant correlation between the anti-oxidant activity resulted from DPPH and FRAP (0.1 < p < 1). There are no previous data on cytotoxic and anti-oxidant activity of A. bicolor since it is an endemic plant. However, anti-oxidant activity as well as cytotoxicity (against NCI-H460, U251, and MCF7) has been reported for A. pavonina, a different plant of the Adenanthera genus [31].

Madhuca longiflora bark extract (methanol), M. pinnata leaf extracts (ethyl acetate and methanol), M. pinnata bark extract (methanol), S. castaneifolia leaf extracts (hexane, dichloromethane, and ethyl acetate), and S. castaneifolia bark extracts (hexane, dichloromethane, ethyl acetate, and methanol) also exhibited potent cytotoxicity against HepG2 cells. The decreased cell volume (compared to control) and morphological appearance of shrunken cells provide evidence for apoptosis in the cells treated with these extracts (3C to 5C of Figure 2). In addition to these morphological changes, it is clear that the percentage of cell viability
Table 2: Cytotoxicity, anti-oxidant activity, total phenolic content, and total flavonoid content of different plant extracts. Cytotoxicity was determined by SRB assay (results are given as IC_{50} value (µg/mL)). Anti-oxidant activity was determined by DPPH and FRAP assays, and results are expressed as EC_{50} (µg/mL) and Trolox equivalents (mg/g), respectively. The total phenolic content and total flavonoid content were determined as mg/g of gallic acid equivalents and mg/g of quercetin equivalents, respectively.

| Plant    | Part   | Extract        | IC_{50} value (µg/mL) and selectivity index (given in brackets) | EC_{50} value (µg/mL) | Trolox equivalents (mg/g) | Gallic acid equivalents (mg/g) | Quercetin equivalents (mg/g) |
|----------|--------|----------------|---------------------------------------------------------------|-----------------------|----------------------------|--------------------------------|-------------------------------|
| Alphalysus cobbe | Leaves | Hexane         | >1,000                                                        | 1,138 ± 1.41          | 5.06 ± 1.72                | 3.52 ± 2.02                    |                               |
|          |        | Dichloromethane| 6.8                                                           | 289.8                | 5.23 ± 4.00                | 123.42 ± 3.93                  |                               |
|          |        | Ethyl acetate  | 19.95                                                         | 273.9                | 7.93 ± 0.06                | 84.24 ± 6.35                   |                               |
|          |        | Methanol       | 11.3                                                          | 269.9                | 8.96 ± 0.56                | 36.78 ± 2.33                   |                               |
|          |        | Hexane         | 288                                                           | 907.30 ± 23.54       | 2.09 ± 1.87                | 2.77 ± 0.15                    |                               |
|          | Bark   | Dichloromethane| 469.3                                                         | >1,000               | 9.43 ± 4.13                | 9.64 ± 1.19                    |                               |
|          |        | Ethyl acetate  | >1,000                                                        | >1,000               | 15.98 ± 3.16               | 3.16 ± 0.89                    |                               |
|          |        | Methanol       | 352.4                                                         | 49.58 ± 6.68         | 82.43 ± 8.60               | 8.6 ± 3.5                      |                               |
| Madhuca longiflora | Leaves | Hexane         | >1,000                                                        | >1,000               | 6.59 ± 0.37                | 4.42 ± 0.44                    |                               |
|          |        | Dichloromethane| >1,000                                                        | >1,000               | 17.23 ± 0.52               | 99.93 ± 9.82                   |                               |
|          |        | Ethyl acetate  | 243.8                                                         | 164.10 ± 13.28       | 202.74 ± 5.52              | 38.51 ± 4.47                   |                               |
|          |        | Methanol       | >1,000                                                        | 0.02 ± 0.01          | 660.77 ± 17.35             | 21.56 ± 3.23                   |                               |
|          |        | Hexane         | 99.49                                                        | 296.8                | 0.43 ± 0.03                | 0.37 ± 0.09                    |                               |
|          | Bark   | Dichloromethane| >1,000                                                        | >1,000               | 5.24 ± 0.54                | 3.16 ± 0.88                    |                               |
|          |        | Ethyl acetate  | 364.8                                                         | 158.22 ± 13.97       | 16.46 ± 3.80               | 8.6 ± 5.28                     |                               |
|          |        | Methanol       | 54.42                                                        | 101.7                | 282.27 ± 23.01             | 1.18 ± 0.01                    |                               |
| Adenothera bicolor | Leaves | Hexane         | 45.86                                                        | 261.1                | 0.56 ± 0.01                | 0.81 ± 0.02                    |                               |
|          |        | Dichloromethane| 27.35                                                        | 182.8                | 1.65 ± 0.59                | 4.67 ± 1.64                    |                               |
|          |        | Ethyl acetate  | 24.56                                                        | 309.1                | 1.55 ± 0.04                | 6.91 ± 2.32                    |                               |
|          |        | Methanol       | 61.83                                                        | 424.5                | 2.29 ± 0.54                | 3.83 ± 1.23                    |                               |
|          |        | Hexane         | 103.7                                                        | >1,000               | 0.29 ± 0.05                | 0.05 ± 0.01                    |                               |
|          | Bark   | Dichloromethane| 45.87                                                        | 69.8                 | 5.42 ± 2.9                 | 3.83 ± 1.23                    |                               |
|          |        | Ethyl acetate  | 236                                                           | 152.13 ± 14.89       | 4.01 ± 3.50                | 3.50 ± 0.30                    |                               |
|          |        | Methanol       | >1,000                                                        | 36.65 ± 10.13        | 345.48 ± 12.92             | 0.53 ± 0.56                    |                               |
| Cylea peltata | Leaves | Hexane         | >1,000                                                        | >1,000               | 2.01 ± 0.94                | 6.78 ± 3.25                    |                               |
|          |        | Dichloromethane| >1,000                                                        | >1,000               | 1.91 ± 0.12                | 1.82 ± 0.83                    |                               |
|          |        | Ethyl acetate  | 113.5                                                        | >1,000               | 1.55 ± 0.04                | 4.64 ± 3.38                    |                               |
|          |        | Methanol       | >1,000                                                        | 476.34 ± 13.34       | 15.20 ± 0.19               | 2.86 ± 3.11                    |                               |
|          |        | Hexane         | >1,000                                                        | >1,000               | 3.41 ± 0.64                | 15.57 ± 5.62                   |                               |
|          | Bark   | Dichloromethane| >1,000                                                        | 765.26 ± 75.95       | 1.41 ± 0.01                |                               |                               |
|          |        | Ethyl acetate  | >1,000                                                        | >1,000               | 5.91 ± 0.46                |                               |                               |
|          |        | Methanol       | >1,000                                                        | 182.57 ± 25.57       | 2.22 ± 1.30                |                               |                               |
| Munronia pinnata | Leaves | Hexane         | 237.6                                                        | >1,000               | 1.27 ± 0.48                | 0.56 ± 0.4                     |                               |
|          |        | Dichloromethane| 269.9                                                        | >1,000               | 14.84 ± 2.56               | 143.32 ± 14.05                 |                               |
|          |        | Ethyl acetate  | 47.07                                                        | 63.9                 | 7.88 ± 0.89                |                               |                               |
|          |        | Methanol       | 56.92                                                        | 287.9                | 2.70 ± 0.22                |                               |                               |
|          |        | Hexane         | >1,000                                                        | >100.0               | 2.70 ± 0.22                |                               |                               |
|          | Bark   | Dichloromethane| 385.4                                                        | 604.89 ± 62.62       | 8.85 ± 3.60                |                               |                               |
|          |        | Ethyl acetate  | 656                                                          | >1,000               | 3.10 ± 0.05                |                               |                               |
|          |        | Methanol       | 52.06                                                        | 139.7                | 10.49 ± 4.64               |                               |                               |
|          |        | Hexane         | 58.42                                                        | 74.61                | 18.54 ± 6.64               |                               |                               |
| Schumacheria castanefolia | Leaves | Hexane         | 39.12                                                        | 12.35 ± 0.10         | 13.00 ± 1.86               |                               |                               |
|          |        | Dichloromethane| 44.5                                                         | 14.88                | 8.06 ± 0.40                |                               |                               |
|          |        | Ethyl acetate  | 198.6                                                        | 0.01 ± 0.01          | 12.08 ± 0.46               |                               |                               |
|          |        | Methanol       | 32.33                                                        | 47.32                | 20.14 ± 3.39               |                               |                               |
|          |        | Hexane         | 4.92                                                        | 50.5                 | 18.54 ± 6.64               |                               |                               |
|          | Bark   | Dichloromethane| <0.01                                                        | 41.7                 | 11.86 ± 1.00               |                               |                               |
|          |        | Ethyl acetate  | 34.14                                                        | 29.5                 | 3.37 ± 0.64                |                               |                               |
Table 2: Continued.

| Plant      | Part   | Extract     | IC_{50} value (μg/mL) and selectivity index (given in brackets) | EC_{50} value (μg/mL) | Trolox equivalents (mg/g) | Gallic acid equivalents (mg/g) | Quercetin equivalents (mg/g) |
|------------|--------|-------------|----------------------------------------------------------------|-----------------------|----------------------------|-------------------------------|------------------------------|
| Carallia   | Leaves | Hexane      | >1,000                                                          | >1,000                | 3.10 ± 0.30                | 2.00 ± 0.20                   | 6.81 ± 2.43                  |
|            |        | Ethyl acetate | >1,000                                                         | >1,000              | 4.40 ± 0.43                | 1.00 ± 0.06                   | 3.50 ± 2.00                  |
|            |        | Methanol    | 228.7                                                          | 101.35 ± 6.77        | 821.05 ± 135.45            | 138.59 ± 6.35                | 10.65 ± 0.70                 |
| Bark       | Dichloromethane | >1,000   | >1,000                                                         | >1,000              | 6.37 ± 2.60                | 6.90 ± 2.26                   | 2.07 ± 1.89                  |
|            |        | Ethyl acetate | 174.3                                                          | 163.33 ± 6.59        | 115.19 ± 18.51             | 11.58 ± 0.82                 | 4.27 ± 2.13                  |
|            |        | Methanol    | 101.5                                                          | 121.59 ± 5.50        | 593.52 ± 88.73             | 2.02 ± 0.54                  | 2.18 ± 1.59                  |

Figure 1: Cytotoxicity of selected active plant extracts against HepG2 cells after 48 h of incubation (dose concentration range: 12.5-200 μg/mL) (a) A. cobbe leaves - hexane extract, (b) A. cobbe leaves - dichloromethane extract, (c) A. cobbe leaves - ethyl acetate extract, (d) A. cobbe leaves - methanol extract, (e) A. bicolor leaves - hexane extract, (f) A. bicolor leaves - dichloromethane extract, (g) A. bicolor leaves - ethyl acetate extract, (h) A. bicolor bark - dichloromethane extract, (i) M. pinnata leaves - ethyl acetate extract, S. castaneifolia leaves extracts, (j) dichloromethane, and (k) ethyl acetate and S. castaneifolia bark extracts (l) hexane, (m) dichloromethane, (n) ethyl acetate, and (o) methanol extracts.
Control

A
50 µg/mL

A
200 µg/ml

B
50 µg/mL

B
200 µg/ml

C
50 µg/mL

C
200 µg/ml

Figure 2: Microscopic observations of HepG2 cells (magnification = ×100) treated with selected active plant extracts (IC₅₀ < 50 µg/mL) after 48 h incubation; 50 µg/mL and 200 µg/mL of (1A) A. cobbe leaves – hexane, (1B) A. cobbe leaves – dichloromethane, and (1C) A. cobbe leaves – ethyl acetate; (2A) A. cobbe – methanol, (2B) A. bicolor leaves – hexane, and (2C) A. bicolor leaves – dichloromethane; (3A) A. boccolor leaves – ethyl acetate, (3B) A. bicolor bark – dichloromethane, and (3C) M. pinnata leaves – ethyl acetate; S. castaneifolia leaves extracts (4A) dichloromethane and (4B) ethyl acetate and S. castaneifolia barks (4C) hexane; and S. castaneifolia barks (5A) dichloromethane, (5B) ethyl acetate, and (5C) methanol. Scale bar 400 µm.

decreased with the increasing concentrations of the identified extracts (Figures 1(i)–1(o)). However, leaf extract of M. pinnata and leaf and bark extracts of S. castaneifolia were also toxic to the normal liver cell line (THLE-3).

Among the extracts studied, methanol extract of leaves and bark of A. cobbe, leaves and bark of M. longiflora, bark of A. bicolor, and leaves of S. castaneifolia had better anti-oxidant activity according to DPPH assay (EC₅₀ value < 100.0 µg/mL) and FRAP assay (>100.0 mg/g of TE) with significance correlation (p < 0.05) and relatively high amount of total phenolic content (>50.0 mg/g of GAE). Oxidants tend to damage DNA and lead to carcinogenesis and uncontrolled cell division. Even though these extracts have strong anti-oxidant properties, they did not exhibit significant cytotoxicity (IC₅₀ value > 100.0 µg/mL) except methanol extract of M. longiflora. Methanol extracts of A. cobbe leaves and M. longiflora bark showed potent cytotoxicity to HepG2 cells with less toxicity to normal cells (IC₅₀: HepG2 = 11.3 and 54.42 µg/mL vs. THLE-3 = 269.9 and 101.7 µg/mL, respectively) and had potent anti-oxidant activity and a considerable quantity of total phenolics. Dichloromethane and ethyl acetate extracts of A. cobbe leaves were cytotoxic to HepG2 cells with less toxicity to THLE-3 (IC₅₀ > 100 µg/mL) and a considerable amount of flavonoid content but anti-oxidant activity.

Hexane, dichloromethane, and ethyl acetate extracts of S. castaneifolia leaves exhibited potent cytotoxicity (IC₅₀ as 35–60 µg/mL) to HepG2 cells after 48 h of incubation. They were toxic to normal liver cells (IC₅₀ 74.61, 12.35, and 14.88 µg/mL for hexane, dichloromethane, and ethyl acetate extracts, respectively, after 48 h exposure). Anti-oxidant activity of these three extracts were considerably low (EC₅₀ > 100 µg/mL for DPPH assay and TE < 40 mg/g for FRAP assay). Hexane extract of S. castaneifolia leaves contained a good quantity of total phenolics (210.24 ± 12.31 mg/g GAE), while dichloromethane and ethyl acetate extracts did not (GAE < 10 mg/g). The total flavonoid content of these three extracts were below 20 mg/g of QE. Even though the methanol extract of S. castaneifolia leaves exhibited less cytotoxicity (IC₅₀ > 150 µg/mL) to HepG2 cells, methanol extract of S. castaneifolia leaves exhibited better anti-oxidant activity (EC₅₀ < 1 µg/mL for DPPH and TE > 800 mg/g for FRAP assays) along with a good quantity of total phenolics (210–230 mg/g of GAE) and a significant amount of total flavonoids (20.14 ± 3.39 mg/g of QE). All extracts (i.e., hexane, dichloromethane, ethyl acetate, and methanol) of S. castaneifolia bark exhibited anti-oxidant activity to both HepG2 (IC₅₀: <35 µg/mL) and normal THLE-3 cells (IC₅₀: 25–55 µg/mL). Hexane and dichloromethane extracts of S. castaneifolia bark had low anti-oxidant activity (EC₅₀ > 300 µg/mL for DPPH and TE < 30 mg/g for FRAP assays) along with the low quantity of total phenols (<5 mg/g GAE) and total flavonoids (<20 mg/g QE). Ethyl acetate extract of S. castaneifolia bark exhibited low anti-oxidant activity (EC₅₀:108.80 ± 11.69 µg/mL and TE > 100 mg/g) with a good quantity of total phenols (>300 mg/g GAE) but a very low amount of total flavonoids (<5 mg/g QE). The methanol extract of S. castaneifolia bark
on the other hand exhibited better anti-oxidant activity by FRAP assay (>1,000 mg/g TE) with a higher content of total phenolic (>300 mg/g GAE).

Although Cylnea peltata has been used in traditional medicine in Sri Lanka, none of the extracts from leaves or bark were cytotoxic to HepG2 cells after 48 h of treatment. Only the methanol extract of C. peltata had some anti-oxidant activity (TE > 150 mg/g in FRAP assay). All the extracts of leaves and barks of Carallia brachiata were less cytotoxic to HepG2 cells (IC_{50} > 100 μg/mL) but had potent anti-oxidant activity. The methanol extract of C. brachiata leaves and ethyl acetate and methanol extracts of C. brachiata barks had some anti-oxidant activity (TE > 100 mg/g in FRAP assay). Of these, the methanol extract of C. brachiata leaves had a higher content of total phenols (>100 mg/g GAE), and the rest of the extracts had a lower content of phenolics and total flavonoids (<50 mg/g of GAE and <50 mg/g of TE, respectively).

Among the extracts of M. pinnata leaves and bark tested, ethyl acetate and methanol extracts of leaves and methanol extract of bark produced better cytotoxic effects (IC_{50} < 60 μg/mL) on HepG2 cells. Unfortunately, the leaf methanol extract was also toxic (IC_{50} 28.79 μg/mL) against THLE-3 cells. All the extracts of M. pinnata had low anti-oxidant activity for DPPH assay (EC_{50} > 300 μg/mL) and a low content of total phenolics (GAE <30 mg/g). Exceptionally dichloromethane extract of M. pinnata had a higher quantity of total flavonoids (>100 mg/g of QE), while all the other extracts of M. pinnata bark had lower flavonoid content (<10 mg/g of QE). Hexane extract of M. pinnata bark showed good anti-oxidant activity only in FRAP assay as TE > 100 mg/g.

Methanol extract of A. cobbe bark, M. longiflora leaves, A. bicolor bark, and S. castaneifolia leaves had higher total phenolic content and potent anti-oxidant activity with less cytotoxicity to HepG2 cells and very low total flavonoid content.

Overall results of the current study suggest that leaves and bark of A. cobbe demonstrated better anti-cancer activity on HepG2 cells with less toxic effects to normal cells. Although several biological functions were previously reported for A. cobbe and other Allophyllus species, none of the studies have reported on their anti-cancer properties [32].

4. Conclusion

In the present study, all the extracts of A. cobbe leaves exhibited potent anti-cancer effects on the hepatocarcinoma cell line with noticeably less toxic effects on normal liver epithelial cells. No relationship was found between cytotoxicity, anti-oxidant activity, and total phenolic and flavonoid contents. It suggests that the anti-cancer activity of A. cobbe leaves is mediated by mechanisms other than anti-oxidant activity. Further studies are needed to isolate active compounds from plants identified to have potent cytotoxic effects and to unravel mechanisms of anti-cancer activity.

Data Availability

The data set supporting this article is included in the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Some traditional medicine practitioners from the Galle district supported the collection of the plants. Plant identification was done by the Royal Botanical Garden of Peradeniya, Sri Lanka. The authors thank technical and IT staff of the Institute of Biochemistry, Molecular Biology, and Biotechnology for their various assistance. The current study was supported by the National Science Foundation (Colombo, Sri Lanka; grant no. RPHS/2016/C.07)

References

[1] A. P. Venook, C. Papandreou, J. Furuse, and L. L. De Guevara, “The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective,” The Oncologist, vol. 15, no. 4, pp. 5–13, 2010.
[2] National Cancer Incidence and Mortality Data Sri Lanka-2012, National Cancer Registry by National Cancer Control Programme, Sri Lanka, 14th Publication, 2020.
[3] G. M. Cragg and D. J. Newman, “Plants as a source of anti-cancer agents,” Journal of Ethnopharmacology, vol. 100, pp. 72–79, 2005.
[4] R. W. Owen, A. Giacosa, W. E. Hull, R. Haubner, B. Spiegelhalder, and H. Bartsch, “The antioxidant/anticancer potential of phenolic compounds isolated from olive oil,” European Journal of Cancer, vol. 36, no. 10, pp. 1235–1247, 2000.
[5] M. Kashiif, D. Kim, and G. Kim, “In vitro antiproliferative and apoptosis inducing effect of a methanolic extract of Azadirachta indica oil on selected cancerous and noncancerous cell lines,” Asian Pacific Journal of Tropical Medicine, vol. 11, no. 10, pp. 555–561, 2018.
[6] D. Bhatia, A. Mandal, E. Nevo, and A. Bishaye, “Apoptosis-inducing effects of extracts from desert plants in HepG2 human hepatocarcinoma cells,” Asian Pacific Journal of Tropical Biomedicine, vol. 5, no. 2, pp. 87–92, 2015.
[7] Y. Cai, Q. Luo, M. Sun, and H. Corke, “Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer,” Life Sciences, vol. 74, no. 17, pp. 2157–2184, 2004.
[8] R. A. Larson, “The antioxidants of higher plants,” Phytochemistry, vol. 27, no. 4, pp. 969–978, 1988.
[9] C. T. Ho, T. Osawa, M. T. Huang, and R. T. Rosen, Food Phytochemicals for Cancer Prevention II: Teas, Spices, and Herbs, American Chemical Society, Washington, DC, USA, 1994.
[10] T. B. Ng, F. Liu, and Z. T. Wang, “Antioxidative activity of natural products from plants,” Life Sciences, vol. 66, no. 8, pp. 709–723, 2000.
[11] K. Xiao, L. Xuan, Y. Xu et al., “Dimeric stilbene glycosides fromPolygonum cuspidatum,” European Journal of Organic Chemistry, vol. 2002, no. 3, pp. 564–568, 2002.
[12] J.-L. Mau, H.-C. Lin, and C.-C. Chen, “Antioxidant properties of several medicinal mushrooms,” Journal of Agricultural and Food Chemistry, vol. 50, no. 21, pp. 6072–6077, 2002.
S. Bains, R. Kaur, and M. Sethi, “Phytochemical analysis of medicinal plants and their antimicrobial activity,” *Agricultural Research Journal*, vol. 57, no. 3, pp. 444–448, 2020.

A. Ghasemzadeh and N. Ghasemzadeh, “Flavonoids and phenolic acids: Role and biochemical activity in plants and human,” *Journal of Medicinal Plants Research*, vol. 5, no. 31, pp. 6697–6703, 2011.

S. H. Baek, L. Cao, S. J. Jeong, H.-R. Kim, T. J. Nam, and S. Samarakoon, C. Shanmuganathan, M. Ediriweera, A. Abbas, S. A. R. Naqvi, M. H. Rasool, A. Noureen, A. Ghasemzadeh and N. Ghasemzadeh, “Flavonoids and phenolic acids; Role and biochemical activity in plants and human,” *Journal of Medicinal Plants Research*, vol. 5, no. 31, pp. 6697–6703, 2011.

M. Madhu, V. Sailaja, T. N. V. S. S. Satyadev, and M. V. Satyanarayana, “Quantitative phytochemical analysis of selected medicinal plant species by using various organic solvents,” *Journal of Pharmacognosy and Phytochemistry*, vol. 5, no. 2, pp. 25–29, 2016.

M. S. M. Saleh, J. Jalil, S. Zainalabidin, A. Y. Asmadi, N. H. Mustafa, and Y. Kamisah, “Genus parkia: phytochemical, medicinal uses, and pharmacological properties,” *International Journal of Molecular Sciences*, vol. 22, no. 618, 2021.

A. Ahmed, A. B. D. Rabou, A. B. Shalby, and H. H. Ahmed, “Anti-cancer activity of Quercetin, Gallic acid and Ellagic acid against HepG2 and HCT116 cell lines: In Vitro,” *International Journal of Pharma and BioSciences*, vol. 7, no. 4, pp. 584–592, 2016.

P. Jayarathna, K. Tennekoon, S. Samarakoon et al., “Cytotoxic, antioxidant and apoptotic effects of twenty Sri Lankan endemic plants in breast cancer cells,” *European Journal of Medicinal Plants*, vol. 15, no. 1, pp. 1–15, 2016.

S. Samarakoon, C. Shanmuganathan, M. Ediriweera, P. Piyathilaka, K. Tennekoon, and I. Thabrew, “Screening of fifteen Mangrove plants found in Sri Lanka for in-vitro cytotoxic properties on breast (MCF-7) and hepatocellular carcinoma (HepG2) cells,” *European Journal of Medicinal Plants*, vol. 14, no. 4, pp. 1–11, 2016.

F. Thovhogi, G. R. A. Mchau, E. T. Gwata, and N. Ntushelo, “Evaluation of leaf mineral, flavonoid, and total phenolic content in spider plant germplasm,” *Molecules*, vol. 263600 pages, 2021.

S. H. Baek, L. Cao, S. J. Jeong, H.-R. Kim, T. J. Nam, and S. G. Lee, “The comparison of total phenolics, total antioxidant, and anti-tyrosinase activities of Korean sargassum species,” *Antioxidant and Antimicrobial Screening of Seriphidium Oliverianum Plant Extracts*, *Dose-Response*, pp. 1–9, 2021.

F. Thovhogi, G. R. A. Mchau, E. T. Gwata, and N. Ntushelo, “Evaluation of leaf mineral, flavonoid, and total phenolic content in spider plant germplasm,” *Molecules*, vol. 263600 pages, 2021.

S. H. Baek, L. Cao, S. J. Jeong, H.-R. Kim, T. J. Nam, and S. G. Lee, “The comparison of total phenolics, total antioxidant, and anti-tyrosinase activities of Korean sargassum species,” *Journal of Food Quality*, vol. 2021, pp. 1–7, 2021.

J. W. Mclarty, “Antioxidants and cancer: the epidemiologic evidence,” in *Antioxidants and Disease Prevention*, H. S. Garewal, Ed., CRC Press, New York, NY, USA, 1997.

E. Niki, “Free radicals, antioxidants, and cancer,” in *Food Factors for Cancer Prevention*, H. Ohigashi, T. Osawa, J. Terao, S. Watanabe, and T. Yoshikawa, Eds., Springer, Tokyo, pp. 55–57, 1997.

C. S. Yang, J. M. Landau, M.-T. Huang, and H. L. Newmark, “Inhibition of carcinogenesis by dietary polyphenolic compounds,” *Annual Review of Nutrition*, vol. 21, no. 1, pp. 381–406, 2001.

S. R. Samarakoon, M. K. Ediriweera, C. D. U. Nwokwu et al., “A study on cytotoxic and apoptotic potential of a triterpenoid saponin (3-O-a-L-Arabinosyl oleanolic acid) isolated from *Schumacheria castaneifolia Vahl* in human non-small-cell lung cancer (NCI-H292) cells,” *BioMed Research International*, vol. 2017, pp. 1–8, 2017.

M. K. Ediriweera, K. H. Tennekoon, S. R. Samarakoon, I. Thabrew, and E. Dilip De Silva, “A study of the potential anticancer activity of *Mangifera zeylanica* bark: evaluation of cytotoxic and apoptotic effects of the hexane extract and bioassay-guided fractionation to identify phytochemical constituents,” *Oncology Letters*, vol. 11, no. 2, pp. 1335–1344, 2016.

I. F. F. Benzine and Y. T. Szeto, “Total antioxidant capacity of teas by the ferric reducing antioxidant power assay,” *Journal of Agricultural and Food Chemistry*, vol. 47, pp. 633–636, 1999.

C. Frezza, F. Sciuoppa, P. Tomai et al., “Phytochemical analysis on the seeds of a new Iranian Plantago ovata Forssk. population specimen,” *Natural Product Research*, vol. 11, no. 379, pp. 1–4, 2021.

S. C. Ghagane, S. I. Puranik, R. B. Nerli, and M. B. Hiremath, “Evaluation of in vitro antioxidant and anticancer activity of Allophylus cobbe leaf extracts on DU-145 and PC-3 human prostate cancer cell lines,” *Cytotechnology*, 2016.

A. J. R. Sophy and A. T. Fleming, “Evaluation of *Adenanthera pavonina* bark extracts for antioxidant activity and cytotoxicity against cancer cell lines,” *International Journal of Science and Research*, vol. 4, no. 12, pp. 2319–7064, 2015.

R. V. Chavan and D. K. Gaikwad, “The Ethnobotany, Phytochemistry and Biological properties of Allophylus species used in traditional medicine: a Review,” *World Journal of Pharmacy and Pharmaceutical science*, vol. 5, no. 11, pp. 664–682, 2017.