Studies on Phytoconstituents, In vitro Antioxidant and Cytotoxicity Activity of Harpullia arborea Bark Extracts on Human Epidermoid Larynx Carcinoma Cell Line (HEp-2)

R. Rajeswari1, S. Murugesh*, D. Jegadeeshkumar2, B. Prakash3, V. Vinoth kumar4
1Department of Botany, School of Life Sciences, Periyar University, Periyar Palkalai Nagar, Salem-636 011, Tamil Nadu, India.
2Chromopark Research Centre, Namakkal - 637001 Tamil Nadu, India.
3Department of Biotechnology, School of Life Sciences, Vels Institute of Science Technology & Advanced Studies, Chennai, Tamil Nadu, India.
4Department of Microbiology, Sree Narayana Guru College, Coimbatore-641105, India.

*Corresponding Author
Email: murugeshss@rediffmail.com
Phone: +91 9943364913
ORCID: 0000-0002-8382-8553

Abstract

Cancer is a major public issue and one of the leading causes of death in prosperous countries. Traditional plants are a valued source of novel cytotoxic agents and are still in performance better role in health concern. In the present study, the bark of methanol and chloroform extracts were subjected to preliminary phytochemicals screening was done using different biochemical tests. Among the 2 solvent extracts, methanol showed the highest number of phytochemicals, which extract was subjected to in vitro study of antioxidant activity by DPPH method and cytotoxicity activity against HEp-2 cell lines by MTT assay. Furthermore, DNA fragmentation and GCMS analysis were subjected. By the analysis, following phyto-constituents like tannins, saponins, Steroids, Carbohydrate and Phenols, Flavonoids, Terpenoids, and quinine were observed. The methanol extract was found to be selectively cytotoxic in vitro to HEp-2 cell lines with IC50 values was 15.6 µg/ml. The GC-MS chromatogram of methanolic bark extract of Harpullia arborea confirmed the presence of various compounds. The present findings strengthen the potential of the selected plant as a resource for the discovery of novel antioxidant and anticancer agents.

Keywords: Anticancer, Harpullia arborea, HEp-2, Catechol, DNA fragmentation.

Introduction

Cancer is a severe metabolic syndrome, and one of the leading causes of death irrespective of developments in disease diagnosis, prevention, and treatment tools. Cancer of the mouth and pharynx ranks the sixth most common cancer in the world population (1). Most of the larynx carcinoma cancers are develops from the squamous cells, which are the majority of the laryngeal epithelium. Cancer can develop in any part of the larynx, but the location of the tumor affects the cure rate (2). The chemical drugs are the toxicity that is caused to the normal cells due to the inability of the chemical drugs to differentiate between normal and cancerous cells. Traditional medicines have been tested and researched to obtain an effective drug against cancer.

Numerous cancer studies for the chemotherapeutic potential of medicinal plants were performed to discover new therapeutic agents that lack the poisonous consequences associated with modern therapeutic agents. Traditional medicine is commonly used as an alternative remedy for most cancers. About 60% of anticancer agents are originated from herbal plants and other natural sources, however, still a few numbers of plants that have anticancer activity, they do not have yet been completely analysis. In recent years, herbal plants and plant acquired products have also attracted researchers due to their varied range of beneficial properties in the management of various cancers (3).

Harpullia arborea (Blanco) Radlk, a native to Indo-Malayan, Western, and Eastern Ghats, belongs to the family Sapindaceae peoples for hair wash, rheumatism and wound healing. The fruit of Harpullia arborea was used as an appetizer and to cure digestive problems (4). The plant is reported to exhibit bioactivities such as larvicidal, antifungal, antioxidant, antibacterial, and anticancer activity (5). Although earlier studies reported curative properties of active phytoconstituents from Harpullia arborea, According to our literature knowledge no one likes that anticancer activity of bark extract of Harpullia arborea against human laryngeal epithelial cell line HEp-2. Thus, the present work intended to screen the methanol extract of Harpullia arborea for testing their potential to inhibit the viability of cells in HEp-2 and determine the antioxidant activity.

Material and Methods

Plant material and extraction

The bark of Harpullia arborea was used in this work, which was collected in the period of April 2017 in Gedamalai, Namakkal District, Tamilnadu, India. The plant was identified at the Botanical survey of India, Coimbatore. The voucher number is BSIS/RC/S/23/2017/Tech/350. The bark sample was...
cleaned and air-dried, and the powder (100gm) was left in chloroform and methanol for 48 h during which the container was stirred occasionally. The content was filtered through 4-fold muslin cloth followed by Whatman filter paper (No. 1). The filtrate was evaporated to dryness. The crude leaf extract obtained was stored in a refrigerator.

Antioxidant study

DPPH radical scavenging assay

DPPH radical scavenging assay was carried out as per the method reported earlier, with slight modifications. Briefly, one ml of the test solution (individual plant extracts) was added to an equal quantity of 0.1 mM solution of DPPH in methanol. After 20 minutes of incubation at room temperature, the DPPH reduction was measured by reading the absorbance at 517 nm. Ascorbic acid (1 mM) was used as the reference compound.

Estimation of total phenolic content

The total phenolic content of Harpullia arboreea was determined using the Folin-Ciochutiu reagent method of Wolle et al., with slight modification. The 200 µl of extract was mixed with an equal volume of Folin-Ciochutiu reagent and incubated for 10 minutes at 37 ºC. Then, 1.25 mL of aqueous sodium carbonate was added, and the reaction mixture and incubated for 90 minutes at 37 ºC after addition of 1mL distilled water. The absorbance of the blue color was read at 760 nm spectrophotometrically using distilled water as a blank. Gallic acid is used as standard, and the total phenolic content was expressed as mg/gm gallic acid equivalent (GAE).

Evaluation of cytotoxicity properties of methanolic extract of Harpullia arboreea

Human laryngeal epithelial cancer cell line (HEp-2) was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were placed into 75 cm² tissue culture flasks and grown at 37 ºC under a humidified, 5% CO₂ atmosphere in Minimum essential medium (Eagle) supplemented with 10% Fetal bovine serum (FBS), 1% glutamine and 100µg/ml penicillin-streptomycin at 37 ºC in 5% CO₂ atmosphere. The HEp-2 oral cancer cells were seeded in 96 well micro titer plates (5X10² cells/well) and 0.1 mL of the test solution (7.8-1000µg) were added to the wells, with the plates kept in an incubator (5% CO₂) at 37 ºC for 72 h. After 72 h, 20µl of MTT was added, and the plates were kept in the CO₂ incubator for two hours, followed by the addition of propanol (100µl). The plates were covered with aluminum foil to protect them from light, and subsequently agitated in a rotary shaker for 10-20 min, after wards the 27-well plates were processed on an ELISA reader to obtain absorption data at 562 nm. The percentage of cytotoxicity compared to the untreated cells. All experiments were performed in triplicates. The morphology of the cells was then examined in an inverted phase contrast microscope.

DCFH-Da staining test for detection of intracellular reactive oxygen species (ROS) level:

Microscopic fluorescence imaging was used to study ROS generation in HEp-2 cells after exposure to different concentrations of extracts. After exposure, cells were incubated with DCFH-DA (10 mM) as the fluorescence agent for 30 minutes at 37ºC. The reaction mixture was aspirated and replaced by 200µl of PBS in each well. The plates were kept on a shaker for 10 minutes at room temperature in the dark. An inverted fluorescence microscope with a CCD cool camera was used to analyze intracellular fluorescence of cells. Increased intensity of intracellular fluorescence was indicative of increased level of generated ROS.

AO/EtBr staining

The AO (0.1 mg/mL) and EtBr (0.1 mg/mL) were used to label nuclear DNA in HEp-2cells. Both solutions were prepared in PBS buffer (pH 7.4). PBS buffer was used to preserve normal physiological activity for unicellular cells. For cell staining, HEp-2 cell samples (100 mL) were stained with AO/EtBr (5 mL) and observed under a fluorescence microscope.

DNA fragmentation assay

The DNA fragmentation assay was carried out based on the method of Sonia and Venkadasan with slight modification. After plant treatment, cells were collected and washed with PBS at 4ºC. Then, cells were centrifuged at 4500rpm for 3min. The obtained pellet was suspended with DNA lysis buffer and incubated on ice for One hr. After incubation 20 µl of RNAse (20 mg ml-1) was added and incubated for One h at 37ºC. Then, 20 µl of Proteinase K (20 mg /ml-1) was added. Follows, the cell suspension were centrifuged at 12,000 rpm for 15min at 4ºC and the supernatant was discarded and transferred to sterile micro centrifuge tube. After, the DNA was precipitated with ice-cold absolute ethanol. Further, the precipitated DNA was washed with 70% ethanol and air dried for 20 min and eventually dissolved with sterile molecular grade water. Same procedure followed for the control cells. The DNA of the sample (15.6µg/ml), control, and DNA ladder were electrophoresed on an ethidium bromide containing agarose gel (1.5%). The gel was visualized and photographed in a gel documentation system.

Statistical analysis

All experiments were performed in triplicate and all data was expressed at least three independent evaluations, and the standard deviations (SD) were also calculated using Microsoft Excel 2008 software.

Identification of bioactive constituents by GC-MS

Samples of methanol extract were further analyzed the by Gas Chromatography — Mass Spectrometry (GC-MS) to determine the species contained in the samples. The chemical components
were identified by matching their mass spectra with those recorded in the mass spectral library. GC-MS analysis was performed by using THERMO GC - TRACE ULTRA VER: 5.0 GC system, MS capillary standard non-polar column, flow rate of 1mL/min, carrier gas was helium, constant flow model. Injector temperature was 260°C, injection volume was 1µL, split injection technique, oven temperature was programmed from 70°C for two min with a temperature increment rate of 6°C/min, and final temperature of 280°C for 2 min. Total running time was 30 min.

**Results and discussion**

Due to their diverse phytometabolic content with various biological activities, the use of herbal medicines in cancer treatment has gained growing attention in recent years. In the present study, plant was collected and identified according to their taxonomic character as *Harpullia arborea*, which was analyzed for the determination of phytochemicals with two solvent extracts.

The Phytochemicals analysis of the methanol extract of *Harpullia arborea* revealed the presence of tannins, saponins, Steroids, Carbohydrate and Phenols. Whereas Flavonoids, Terpenoids, and quinine were present in chloroform extract. The presence of Tannin and Terpenoids were observed in both extracts. In 2010, previous study also observed the saponins and steroids from the seed of methanol extracts of *Harpullia arborea*.[11] The obtained result in this study is the first report on the bark extract of *Harpullia arborea*.

Among the different phytochemicals, phenolic compounds have been brought to the attention of various applications such as pharmaceutical, health, food, and cosmetic industries. As part of our daily diet, these compounds are abundant in the plant kingdom and attractive as natural antioxidants[10], anticancer agents[11]. In this present study, the highest phyto constituents were observed from methanol extract, therefore which was subjected for further study. Earlier reported that seed extract had saponins, resins, glycosides, and steroids as the chemical class present in the extracts[11].

Antioxidant activity plays a key role in reducing chronic diseases such as cancer and cardiovascular disease (CAD). DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The removal of DPPH solution increases linearly with increasing amount of extract in a given volume. The DPPH method revealed that the scavenging of the free radicals was found to be 10.2 ±0.78%, 21.21±1.62%, 41.01±3.12%, 59.13±4.53% and 75.14±5.75% at 1, 2, 3, 4 and 5 mg/ml respectively.

The antioxidant capacity of the plant extract was correlated with ascorbic acid. The scavenging of the free radicals was found to be 15±1.14%, 30.42±2.33%, 55.11±4.2%, 76.34±5.84% and 85.14±6.52% at 1, 2, 3, 4 and 5mg/ml respectively. In the DPPH assay, the IC₅₀ of ascorbic acid was 2.8 mg/ml while that of the bark extract was 3.5 mg/ml (Fig 1). The scavenging ability of this extract was less than those of ascorbic acid. This present study showed that the extract has proton donating ability and could serve as free radical inhibitors, possibly acting as primary antioxidants. Earlier report[4] found the antioxidant activity of leaf extract of *Harpullia arborea*. Mostafa et al. [13] observed the DPPH radical scavenging potential from two *Harpullia* species viz. *H. cupanioides* and *H. pendula*.

The analysis of the cytotoxicity activity of plant extract is important for safe treatment. The antioxidant assay results further, directed the study towards cytotoxic assay. The results of the cytotoxicity of a selected plant (methanol extract) against the HEp-2 cancer cells are summarized in Fig 2. Results were reported as the percent growth of the treated cells when compared to the untreated control cells. It could also specify an indication of possible cytotoxic properties of the tested plant extract of *Harpullia arborea*. MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase and the entire extracts exhibit a gradual increase in antiproliferative activity with respect to dose dependent manner.

The highest cytotoxicity of this extract against HEp-2cell was found in 200 and 100 µg/ml concentrations with 97.58 and 88.92 percent of cell growth inhibition. It was found that the percentage of growth inhibition to be reducing with increasing of test compounds, and IC₅₀ value of this assay was 15.6 µg/ml. According to a review of literature, no one report about the anticancer activity of bark extract of *Harpullia arborea* against HEp-2 cells. Seham et al (14) reported that same genus of (*Harpullia cupanioides* Roxb) and *Harpullia pendula* Planch. Ex F.Muell) plants were capable to active against various cancer cells. As far as we are aware, this is the first time to refer to the in *vivo* cytotoxic activity of *Harpullia arborea* bark extract against the HEp-2 cancer cell lines under investigation (Fig 3).

The Fig. 4 was illustrated that plant treated cancer cells stained with DCFH-DA become more fluorescent with increased dosage indicative of significant intracellular ROS accumulation inducing apoptosis. Cells treated with extract emitted bright fluorescence with cripple morphology because of disturbance in the integrity of plasma membrane due to ROS generation.

Furthermore, Plant extract treated HEp-2 cells were subjected to AO/EB staining. AO stain enters the nucleus and marks the nuclei green, and EB will penetrate the nucleus of dead cells, is mainly taken up by the cells when membrane integrity is lost and stains the nuclei red. Since AO intercalates in the DNA but only interacts with the RNA, viable cells do not uptake EtBr and these
cells exhibit green nuclei. In the present study, early apoptotic cells were appeared as a granular yellow-green with AO nuclear staining and necrotic cells revealed uneven, orange-red fluorescence at their periphery without chromatin fragmentation (Fig. 5).

In this study, DNA fragmentation assay was carried out with the plant extract for elucidating the mechanism of cells death. The DNA fragmentation clearly demonstrates the role of apoptosis in plant treated cells. The DNA migrated as separate bands which were compared to DNA markers and untreated cell. From this it is revealed that this DNA fragment shows that the methanolic extract of Harpullia arborea has anticancer activity in the HEp-2 cell lines. According to previous studies, no one likes was reported that DNA fragmentation of HEp-2 cell line with Harpullia arborea (Fig. 6).

From this study showed antioxidant and cytotoxicity of tested plant extract against HEp-2 cells, which indicate there could be some phyto compounds in this extract. Several authors reported that phenolic acids, flavonoids, steroids, terpenoids are known to be bioactive principles. Baba and Malik (15) was reported that close relationship between total phenolic contents and antioxidative activity of plants. In this present study, phenolic content was studied in the bark extract of Harpullia arborea, where in the methanolic extract exhibited 35.20±0.14mg/gm GAE.

In this present study, GC-MS chromatogram of methanolic bark extract of Harpullia arborea showed number of peaks, which indicates the presence of various compounds. The mass spectral fingerprint of each compound can be identified from the data library. From the chromatogram peaks, found different bioactive leads which include phenolic compounds and fatty acid and the detailed of each compound was listed in Table 1. Among them, following cancer inhibitory substance were observed including catechol, 1, 2, benzene dicarboxylic acid, and Phthalic acid. According to a review of literature no one was found the anticancer activity of catechol from Harpullia arborea.

The catechol, also known as pyrocatechol, is a naturally-occurring compound found in fruits and vegetables such as onions, apples and olive oil (16 % 17). This was showed anti-cancer activity by directly binding with signaling molecules important in carcinogenesis. The previous report (18) of were inhibited the lung cancer cells and latterly (19) were reported the anticancer against Human HepG-2 cancer. However, no report has yet explained the anticarcinogenic effects of catechol against HEp-2 cancer cells. In view of this study, catechol may be inhibited the HEp-2 cells and exhibiting anti-cancer properties.

In conclusion, it was observed that the plant of Harpullia arborea consists of a broad range of secondary metabolites that hold robust antioxidant and anticancer capability based on the experiments performed which add scientific evidence to conduct in addition research, investigate the phyto compounds current in the plant, consider its anticancer achievable on in vivo animal model and put forward a try to carry out trails on human beings.

Conflicts of interest
The authors declare that they have no competing interests.

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| 11    | 15.591 | 15.509 | 0.38   | 1.00   | 3.07    | V 2-BENZENEDIACETIC ACID, DICHE 
| 12    | 16.562 | 16.531 | 0.62   | 0.87   | 2.98    | V 1-Decenedioic acid, 1-decyl ester |
| 13    | 17.455 | 17.417 | 0.05   | 0.18   | 2.33    | V 1-Decenedioic acid, 1-decyl ester |
| 14    | 18.190 | 18.090 | 0.24   | 0.41   | 3.06    | V 2,6-DIMETHYLCYCLOHEX-2-EN-1-OL, 1-OCTYL ester |
| 15    | 18.737 | 18.700 | 0.04   | 0.15   | 2.55    | V Phthalic acid, butyl octyl ester |
| 16    | 19.185 | 19.142 | 0.11   | 0.34   | 2.85    | V OCTACARBOXYLIC ACID, METHYL ESTER |
| 17    | 19.753 | 19.708 | 0.07   | 0.21   | 2.89    | V Phthalic acid, butyl octyl ester |
| 18    | 20.642 | 20.608 | 0.09   | 0.18   | 4.22    | Vis-Phthalic acid, butyl octyl ester |
| 19    | 21.039 | 20.958 | 0.13   | 0.29   | 3.83    | V 8-OCTADECENOIC ACID, 2-METHYL- |
| 20    | 21.385 | 21.256 | 0.56   | 0.71   | 3.22    | V 5-HEXADENOIC-1-OL, 3,7,11-TRIMETH |
Fig. 1 DPPH radical scavenging activity

Fig. 2 In vitro Cytotoxicity of Methanolic bark extract of Harpullia arborea against HEp-2 Cell line

A

B

C

D

Fig. 3 Morphological changes of HEp-2 cell lines after MTT assay treatment. A - 200µg of extract, B - 50 µg of extract, C - 12.5 µg of extract, D - Control (Without plant treatment).
Fig. 4 DCFH-DA staining test for detection of MECD induced intracellular ROS level: Control - Healthy HEp2 cancerous cells without fluorescence (no ROS), Low- Extract treated cells with less fluorescence, IC50- Extract treated cells with more fluorescence.

Fig. 5 Hep2 cells showing red fluorescence indicated that they are undergoing apoptosis with Acridine Orange/Ethidium Bromide stain and control cells showed green fluorescence

Fig.6 DNA fragmentation of plant extracts, M- 1KB DNA marker, 1-Control cells, 2-Low concentration of extract, 3- IC50 Concentration

References


