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Research Article

MTT-Based Evaluation of HepG2 Cell Cytotoxicity Induced by a Polyherbal Extract of Medicinal Plants with Reported Anticancer Properties

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ABSTRACT

Hepatocellular carcinoma (HCC) is a highly aggressive liver cancer with a poor prognosis, necessitating timely diagnosis and effective treatment strategies. Conventional therapies, including liver transplantation, chemotherapy, targeted drugs, and herbal remedies, provide limited curative outcomes and mainly aim to extend survival and alleviate symptoms. This study investigated the anticancer activity of a polyherbal formulation on HepG2 cell lines. The preparation consisted of hydro-alcoholic and ethanolic extracts of *Andrographis paniculata*, *Annona muricata*, *Eclipta alba*, and *Aegle marmelos*, all recognized for their anticancer, antioxidant, and hepatoprotective properties. Assessment *via* cell viability assays revealed a significant reduction in cancer cell survival upon treatment. The observed effects are likely due to synergistic interactions among the extracts, targeting multiple cancer-associated pathways such as apoptosis induction, inhibition of cell proliferation, and reduction of oxidative stress. These findings suggest the potential of polyherbal formulations as complementary therapies for HCC. However, further *in-vivo* and clinical studies are necessary to confirm efficacy, determine safe and effective dosages, and establish comprehensive safety profiles. This work highlights the importance of integrating traditional herbal knowledge with modern research to develop innovative strategies for HCC management.

Introduction

The liver, an indispensable organ body, is fundamentally involved in numerous biochemical processes. It is responsible for detoxifying harmful substances and synthesizing essential compounds, underscoring its critical physiological functions. Consequently, liver damage can have severe consequences.[1] In recent years, research has increasingly focused on developing new treatments that can minimize the adverse effects associated with conventional cancer therapies. This shift highlights the growing interest in identifying more targeted and less toxic therapeutic approaches to enhance patient outcomes and quality of life. Nature has long provided medicinal substances, with many modern drugs derived from authentic sources, regularly drawing inspiration from traditional medicinal knowledge.[2,3] Herbal compounds, in particular, have served as a valuable source for drug discovery against various diseases. Numerous medicinal plants have demonstrated positive effects in both experimental and clinical cancer chemoprevention and chemotherapy studies. Since complex diseases like cancer involve multiple pathways and complications, their management often requires more than one therapeutic agent. Thus, combining anticancer herbs, rather than relying on single-herb therapy, may offer improved clinical outcomes.[4]

In this context, a polyherbal formulation comprising *Annona muricata*, *Andrographis paniculata*, *Aegle marmelos*, and *Eclipta alba* is proposed for liver cancer treatment.

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Table 1: Bioactive compounds and mechanistic effects of selected medicinal plants in liver protection and hepatocellular carcinoma

Plant	*Major active compounds*	*Mechanisms*	*References*
Annona muricata	Acetogenins (annonacin, bullatacin), alkaloids	Antioxidant, apoptosis induction, inhibition of DEN-induced hepatocarcinogenesis	[19, 20]
Andrographis paniculata	Andrographolide, neoandrographolide	Anti-inflammatory, Nrf2 activation, STAT3/NF-κB suppression, apoptosis in HCC cells	[21, 22]
Eclipta alba	Wedelolactone, demethylwedelolactone, luteolin	Antioxidant enzyme activation, anti-inflammatory, Wnt/β-catenin inhibition	[23, 24]
Aegle marmelos	Marmelosin (imperatorin), aegeline, lupeol	Antioxidant, DNA protection, suppression of DEN-induced hepatic cancer	[25, 26]

Annona muricata and other selected plants contain bioactive compounds with significant anticancer potential. Indigenous to tropical and subtropical regions, extracts of *A. muricata* are particularly recognized for their ability to combat cancerous cells.[5] Graviola (*A. muricata*) extract has been reported to exhibit antioxidant, chemopreventive, and chemotherapeutic effects, effectively suppressing DMBA-induced mammary carcinogenesis in Swiss albino mice.[6] Its ethanolic leaf extract (AMLE) has also been shown to reduce DMBA/croton oil-induced skin papilloma formation. [7] Additionally, the extract inhibited Ehrlich ascites carcinoma growth, restored hematological parameters, and provided protection against benzo[a] pyrene-induced lung tumorigenesis. [8]

Another important compound, andrographolide derived from *A. paniculata* is widely recognized for its diverse pharmacological activities.[9] The ethanolic extract of *A. paniculata* significantly improved colon tissue architecture and decreased the frequency of abnormal crypt lesions (ACF) in DMH/HFD-induced colon carcinogenesis in rats. [10] Andrographolide, the major bioactive constituent, has demonstrated notable anticancer effects, including cell cycle blockage in LoVo human colorectal carcinoma cells [67] and the hindrance of cancer cell growth. [11]

The ethanolic fruit pulp extract of *Aegle marmelos* showed antiproliferative activity by slowing breast tumor growth in rat models, while also exerting hepato-renal protective effects.[12] Cell-culture studies have revealed that *A. marmelos* Correa extracts inhibit the proliferation of various human tumor cell lines, such as K562 (leukemia), Jurkat (T-lymphoid), Raji (B-lymphoid), HEL (erythroleukemia), Colo38 (melanoma), and the breast cancer lines MCF-7 and MDA-MB-231.[13] Bioactive phytochemicals found in various parts of *A. marmelos* (fruits, bark, leaves, seeds, and roots) have also been documented for their anticancer properties.[14] Similarly, the methanolic extract of *Eclipta alba* exhibited strong activity against colorectal cancer HCT-116 cells while showing minimal toxicity toward normal WI-38 cells.[15] Its alcoholic extract further revealed significant can help combat breast cancer cells by causing them to undergo apoptosis and preventing them from multiplying, migrating, and demonstrating potent antioxidant activity, with low toxicity observed in animal studies highlighting its value as a safe complementary anticancer agent.[16] *E. alba* constituents have also traditionally been used for anticancer purposes and have shown *in-vitro* activity against various cancer cell lines. [17] The hepatoprotective and anticancer effects may be attributed to phytochemicals such as acetogenins from *A. muricata*, andrographolide from *A. paniculata*, wedelolactone from *E. alba*, and marmelosin from *A. marmelos*, which modulate oxidative stress, inflammation, and apoptosis pathways, thereby protecting against hepatic injury and carcinogenesis (Table 1).

Since HepG2 is widely accepted as a model, this cell line is used for liver cancer research and is crucial for determining the cytotoxicity of natural extracts. It is imperative to evaluate the cell viability of these selected plant extracts using the *in-vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Although substances isolated from *A. muricata*, *A. paniculata*, *A. marmelos*, and *E. alba* have revealed significant anticancer, antioxidant, and protective attributes in different cancer models, there remains a considerable research gap in understanding their precise mechanisms, efficacy, and safety in liver cancer, where existing treatment options are still limited and often associated with toxicity and resistance. One widely used technique to measure cell viability and cytotoxicity is the MTT assay. However, interference by plant ethanol extracts may sometimes result in false-positive viability outcomes. To ensure reliable evaluation of the cytotoxic effects of these extracts, this study specifically investigates the accuracy of the MTT assay when applied to plant-based formulations. [18]

This study holds pharmaceutical significance by assessing the cytotoxic effects of a polyherbal formulation on HepG2 cells using MTT assays. Given the limitations of current HCC therapies in terms of efficacy, toxicity, and cost, plant-based formulations represent a promising alternative or adjunct treatment. Investigating combined extracts with known anticancer properties may reveal synergistic effects that target multiple cancer pathways, providing a foundation for developing safer and more effective therapeutic strategies that integrate traditional herbal knowledge with modern pharmacology.

Materials and Methods

Chemicals and Reagents

A basic nutrient medium supplemented with fetal calf serum for cell line maintenance, and ethanol for the extraction of plants was used. The standard drug used here was obtained from local pharmacists.

Plants

The plant materials, in powdered form, were collected in October 2024 and were procured and authenticated from Sanchomee Herboveda Pvt. Ltd. (trading as Manikarnika Aushadhalaya, Chinchwad, Pune, India). A voucher specimen (No. SHPL/03/23) is maintained in the Department of Pharmacology, Dr. D. Y. Patil College of Pharmacy.

Cell Culture

HepG2 cell lines were maintained at the Scitesla Research Organization, Navi Mumbai. The cell lines were cultivated in a basic nutrient medium (MEM) supplemented with fetal bovine serum (FBS).

Methods[27]

Crude plant powder was obtained from Sanchomee Herboveda Pvt. Ltd (Chinchwad), and all the plants were in the form of coarse powder. The procedure for extracting the extract is explained in Fig. 1.

Statistical Analysis

All experiments were done three times, and the results are shown as the average ± standard deviation (SD). Differences between groups were tested using one-way ANOVA with Tukey's post hoc test. Results with a *p-value* less than 0.05 were considered significant, with significance levels shown as $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$.

Table 2: Plants used in polyherbal extract

Plant name	*Family*	*Plant part*
Annona muricata	Annonaceae	Leaves
Andrographis panniculata	Acanthaceae	Bark
Aegle marmelos	Rutaceae	Leaves
Eclipta alba	Asteraceae	Leaves

Phytochemical Evaluation

A preliminary phytochemical investigation was carried out on the crude extracts of *A. muricata*, *A. paniculata*, *A. marmelos*, and *E. alba*. The extracts were examined to identify the main types of secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, and phenolic compounds, using standard qualitative methods. Procedures, as outlined in recognized phytochemical testing manuals, were employed for the screening of these constituents.

No further purification, fractionation, or quantitative determination, such as total phenolic or flavonoid content estimation, GC–MS, or HPLC profiling, was undertaken. The evaluation was confined to crude extracts owing to the preliminary and exploratory nature of the work as well as limited analytical resources. This screening was intended to provide an initial understanding of the phytochemical composition that may contribute to the biological potential of the plant extracts.

Cell Culture Preparation

Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) to provide nutrients and growth factors. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 to mimic physiological conditions for optimal growth.

Seeding in a 96-well Plate

Cells were plated in a 96-well plate, which is commonly used for high-throughput analyses. A uniform seeding density of 1×10^5 cells per well was used to ensure consistent distribution and standardized experimental conditions. Following seeding, 100 µL of MEM was added to each well to fully immerse the cells in culture medium. The plate was then incubated for 24 hours at 37°C with 5% CO_2 to allow adequate cell attachment and stabilization before treatment.

Introduction of Test Extract

Following the initial incubation period, the medium in each well was gently removed and replaced with the test extract prepared in the same base medium. This step ensured that any observed effects were due to the test extract rather than residual components of the initial medium. Cells were applied at different doses of the extract (25,

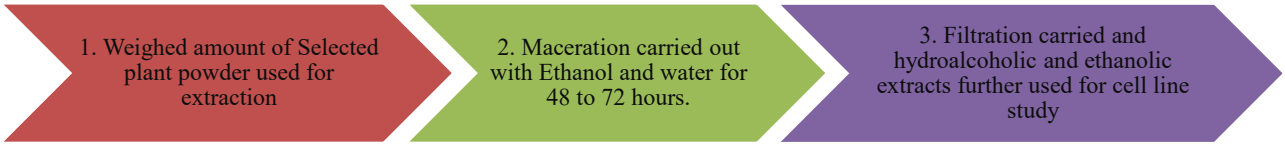

Fig. 1: Extraction process for selected plant materials

50, 100, 250, 500, and 1000 µg/mL) for 24 hours. The test concentrations were selected based on the effective range reported for traditionally used medicinal plant extracts in previous *in-vitro* anticancer studies.[28] The MTT assay was used to measure cell viability. The treated plate was then incubated for an additional 24 hours under the same conditions. This allowed the cells to interact with the test extract, enabling the investigation of its effects on cell viability, proliferation, or any other cellular response.

MTT ASSAY

MTT assay setup

Following the incubation with the test extract, 100 µL of the MTT reagent (prepared at 5 mg/mL) was introduced into each well of the 96-well plate. MTT, a yellow tetrazolium salt, is widely used in cell viability assays as mitochondrial dehydrogenases metabolically reduce it in living cells into insoluble formazan crystals. This reaction reflects metabolic activity as an indirect measure of viability. The plate was then returned to the incubator and incubated for four hours at 37°C to allow sufficient time for the enzymatic conversion of MTT into formazan by viable cells. After incubation, the supernatant (remaining liquid above the crystals) was carefully discarded from each well, ensuring the formazan crystals remained intact.

Formazan solubilization and optical density measurement

To achieve complete solubilization of the insoluble formazan crystals, 100 µL of a solubilizing agent (such as DMSO, isopropanol, or a specialized reagent) was added to each well. This step ensured complete solubilization of the crystals, resulting in a homogenous, colored solution. The absorbance of each well was then measured at a wavelength of 570 nm using an EPOCH microplate reader (manufactured by BioTek-Agilent, USA). The optical density (OD) values obtained correspond to the color produced, serving as an indicator of the number of viable cells with active metabolism in each well.

Table 3: Cytotoxic effect of the polyherbal extract and standard drug on HepG2 Cells (MTT assay)

S. No.	*Sample*	*Concentration (µg/mL)*	*%Cell viability (Mean ± SD)*
1	Polyherbal extract	25	125.37 ± 0.85
2	Polyherbal extract	50	62.77 ± 0.67 ***
3	Polyherbal extract	100	41.63 ± 0.38 ****
4	Polyherbal extract	250	38.36 ± 0.46 ****
5	Polyherbal extract	500	38.52 ± 0.25 ***
6	Polyherbal extract	1000	36.55 ± 0.25 ****
7	Standard drug	25	347.20 ± 1.20
8	Standard drug	50	76.40 ± 0.44 ***
9	Standard drug	100	63.43 ± 0.25 ****
10	Standard drug	250	45.57 ± 0.25 ****
11	Standard drug	500	37.03 ± 0.23 ***
12	Standard drug	1000	36.03 ± 0.15 ****

RESULT

The cytotoxic activity of the polyherbal extract and the standard drug against HepG2 cells was assessed using the MTT assay across concentrations ranging from 25 to 1000 µg/mL. As shown in Table 2**,** both samples exhibited a clear dose-dependent reduction in cell viability.

At the lowest concentration (25 µg/mL), the polyherbal extract showed 125.37 ± 0.85% viability, indicating minimal cytotoxicity (Table 3). However, cell viability decreased significantly with increasing concentrations, reaching **36.55 ± 0.25% (**$p < 0.0001$) at 1000 µg/mL. Similarly, the standard drug demonstrated a comparable pattern, with viability reducing from 347.20 ± 1.20% at 25 µg/mL to **36.03 ± 0.15% (**$p < 0.0001$) at the highest concentration.

Statistical evaluation using one-way ANOVA with Tukey's post hoc test showed that each treatment group differed significantly from the control ($p < 0.05$). Both treatments exhibited strong, concentration-dependent cytotoxic effects, and the polyherbal extract displayed efficacy comparable to the standard drug at higher concentrations, indicating its promise as a naturally derived therapeutic agent candidate against hepatocellular carcinoma.

DISCUSSION

The current study investigated the cytotoxic potential of a polyherbal formulation composed of *A. muricata*, *A. paniculata*, *A. marmelos*, and *E. alba* against HepG2 liver cancer cells using the MTT assay. The results revealed a clear concentration-dependent decrease in cell viability, indicating strong cytotoxic activity of the polyherbal extract comparable to the standard drug at higher concentrations. The observed reduction in viability from 125.37 ± 0.85% at 25 to 36.55 ± 0.25% at 1000 µg/mL suggests a potent antiproliferative effect, which may result from synergistic interactions among the bioactive constituents present in the selected medicinal plants.

These findings are in agreement with previous studies reporting the anticancer potential of the individual plant components. *A. muricata* contains acetogenins such as annonacin and bullatacin that exhibit strong cytotoxic and pro-apoptotic activities by inhibiting mitochondrial complex I, leading to energy depletion and cancer cell death.[5-8] *A. paniculata* possesses andrographolide, a diterpenoid lactone known to activate Nrf2 signaling, suppress NF-κB and STAT3 pathways, and induce apoptosis in hepatocellular carcinoma and colon cancer models. [9-11,21,22] Likewise, *E. alba* has been documented to exert hepatoprotective and anticancer effects through its coumestan derivatives such as wedelolactone and demethylwedelolactone, which modulate antioxidant

defense and inhibit the Wnt/β-catenin pathway.[15-17,23,24] *A. marmelos* contributes active constituents like marmelosin, aegeline, and lupeol, which display antioxidant, anti-inflammatory, and DNA-protective effects, along with suppression of diethylnitrosamine (DEN)-induced hepatic carcinogenesis.[12-14,25,26] Collectively, these bioactive molecules likely act on diverse signaling mechanisms that govern oxidative stress, inflammation, and apoptosis, resulting in the enhanced cytotoxic efficacy observed in this study.

The dose-dependent cytotoxicity observed against HepG2 cells suggests that the polyherbal extract disrupts mitochondrial function and promotes apoptosis, consistent with mechanisms previously reported for the individual herbs.[5-11,23] Similar trends have been documented for *A. muricata* and *A. paniculata* extracts, which induce apoptosis and promotion of cell-cycle blockade in breast, colon, and various hepatoma cell lines.[6,10,11] The significant inhibition of cell viability at concentrations above 100 µg/mL reinforces the potential of this polyherbal formulation as a candidate for liver cancer therapy.

However, this investigation was limited to qualitative phytochemical evaluation of crude extracts. No fractionation, chromatographic separation, or quantitative estimation of total phenolic and flavonoid contents was conducted. This limitation is primarily due to the exploratory nature of the study and resource constraints. Nevertheless, the preliminary results provide an essential foundation for future research aimed at isolating, characterizing, and quantifying the specific phytoconstituents responsible for the cytotoxic effect. Advanced analytical techniques such as GC–MS, HPLC, or LC–MS can further confirm the active compounds and their relative abundance in the extract.

Additionally, although the MTT assay is a standard process to assess cytotoxicity, it is known that plant extracts—especially ethanolic ones—can sometimes interfere with colorimetric readings due to their inherent pigments or reducing components.[18] Despite this, the present data were consistent, statistically significant ($p < 0.05$), and reproducible across triplicates, confirming the reliability of the observed cytotoxic effect.

Overall, the findings support the hypothesis that combining medicinal plants with complementary mechanisms of action may yield greater anticancer activity than individual plant extracts. The polyherbal combination exhibited strong cytotoxicity against HepG2 cells, supporting its suitability as a natural bioactive agent candidate for hepatocellular carcinoma. Further mechanistic studies—such as evaluation of apoptosis markers (caspase activation, mitochondrial membrane potential), oxidative stress parameters, and gene expression profiling—along with animal-based studies, are warranted to substantiate the efficacy and safety of this polyherbal formulation.

Conclusion

The polyherbal formulation containing *A. muricata*, *A. paniculata*, *A. marmelos*, and *E. alba* demonstrated significant, dose-dependent cytotoxic activity against HepG2 liver cancer cells. The observed effects may be attributed to the synergistic action of diverse phytochemicals that target multiple cancer-related pathways. Although the study was limited to crude extract evaluation, it provides a scientific basis for further isolation and mechanistic studies to develop a safe, plant-based therapeutic option for hepatocellular carcinoma.

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