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In vitro antiproliferative potential of *Cassia angustifolia* extracts on HepG2 cells to combat liver cancer

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Abstract

Liver cancer is a terrifying disease with limited treatment options and is responsible for global health and economic burden. Most anticancer options have severe adverse effects, including non-specific modes of action that aggravate the challenges of exploring effective, safe, and economical therapeutic sources. The current study aimed to decipher the cytotoxicity of ethanol (ECA) and *n*-hexane (HCA) extracts of a potential medicinal plant *Cassia angustifolia* on human liver cancer (HepG2) and noncancer human embryonic kidney (HEK-293) cells. *Cassia angustifolia* extracts were investigated first to profile phytocompounds through gas chromatography-mass spectrometry (GC-MS). Varying concentrations of *C. angustifolia* extract (10, 50, 100, 200, and 400 µg/mL) were evaluated for cytotoxicity through the MTT assay, cell viability assay, and morphological examination using a Floid cellular imaging station. Results of GC-MS analysis identified 45 compounds in ECA and HCA extracts, from which 10 compounds were common in both extracts. Most of them are reported to have antiproliferative activity. The ECA and HCA extracts showed potent cytotoxicities (IC_{50}= 62 and 67 µg/mL) in HepG2 cells, while minimal effects on noncancer HEK-293T cells (IC_{50}= 245 and 482 µg/mL) were recorded. The viability of cancer (HepG2) cells was calculated to be 98%, 79%, 45%, 30%, and 25% for ECA and 95%, 68%, 55%, 40%, and 31% for HCA extract, respectively. Significant percent viabilities on noncancer (HEK293T) cells were observed to be 95%, 90%, 88%, 76%, and 61% with ECA extract, and 97%, 95%, 88%, 75%, and 62% with HCA extract compared to those of untreated (UT) (100%) and DMSO (100%) treated cells. Cisplatin (positive control) exhibited <40% and <38% viabilities of cancer and normal cells, respectively. Morphological examination revealed that proliferation was reduced at 100, 200, and 400 µg/mL of the ECA extract and 200 and 400 µg/mL of the HCA extract on HepG2 cells. The HEK-293T cells did not show a noticeable decrease in viability by both extracts. In conclusion, *C. angustifolia* extracts showed considerable anticancer activity due to bioactive phytocompounds and were comparable to cisplatin (an FDA-approved drug). After further isolation and validation, the identified phytocompounds can be available as safer anticancer candidates against liver cancer.

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Introduction

Cancer is characterized by the uncontrolled proliferation of subtly altered normal human cells. The overall mortality rate is predicted to increase from one million in 2020 to more than 1,6 million in 2050, causing a significant global health and economic burden. Globally, hepatocarcinoma (HCC) ranks sixth among the most prevalent cancers, with approximately 4.7% (841,000) new cases and 8.2% (782,000) deaths due to HCC in 2018 (Bray et al., 2018). Over the past 40 years, the cases of HCC have increased three times in the United States (USA), maybe due to the maturation of chronic hepatitis C (CHC) infection in the population. In East Asia, chronic hepatitis B patients had a 0.2% frequency of developing HCC, whereas cirrhosis patients had 3.7%. Chronic HCV infection is nearly 17 times more prone to develop HCC, the primary cause of most HCC in developed nations (Samant et al., 2021).

Intervention strategies are needed, such as chemo-preventive medications that affect early cancer initiation and act by delaying, preventing, or reversing epithelial-mesenchymal transition and acting as anticancer therapeutic agents (Khalil et al., 2015). The use of chemotherapeutic drugs for cancer is a promising area, which is engaged in reducing the effects of carcinogenic agents and have low toxicity while being highly effective for reducing tumor growth. The search for naturally occurring anticancer drugs has recently received more attention from the scientific community because of their bioactive components. Most of these bioactive chemicals influence signal transduction pathways contributing to the induction of apoptosis, progression of cell events, and proliferation. Literature has shown that herbal treatments are more effective and have lesser adverse effects than those of synthetic/allopathic medications (Al-Dabbagh et al., 2018).

The significance of plants in conventional medicines used as raw stuff in pharmaceutical industries is at the highest level. Phytocompounds, which have tremendous therapeutic potential in medicinal plants, offer a revolutionary advantage because of their hidden potential against various ailments, including cancer (Bisht et al., 2021). Polyphenols comprise a large group of naturally occurring antioxidants and volatile compounds that can protect against cardiovascular diseases, inflammations, neurodegenerative disorders, and cancers. The health benefits conferred by flavonoids, a class of secondary metabolites, are attributed to antioxidant activity in bio-signaling pathways (Pinto et al., 2021). Numerous *in vitro* investigations have described the anticancer properties of these compounds by activating DNA repair stimulation, enzymatic inhibition, antioxidant mechanisms, and releasing protective enzymes. Some phytochemicals show significant clinical results based on their toxicity, efficacy, pharmacokinetics, safety data, and approval in cancer treatment, such as vinca alkaloids or paclitaxel (Roy and Bharadvaja, 2017). GC-MS is one of the best approaches to profile the phytoconstituents for identifying novel biologically active compounds like polyphenols, alkaloids, saponins, terpenes, and flavonoids from plant extracts (Talib and Aftab, 2021).

Cassia angustifolia, (also known as Senna) is highly acknowledged in the Pharmacopeia of the USA because of its therapeutic effects on typhoid, anemia, toxicity, and cholera (Albrahim et al., 2021). Its leaves and seeds have abundant pharmacological characteristics, including antibacterial, anticancer, antifungal, anti-inflammatory, antioxidant, antidiabetic, hepatoprotective, and hypercholesterolemia. *Cassia angustifolia* maintains hepato-protective activity by regulating liver enzymes in case of liver injury. The high consumption of *C. angustifolia* demands isolation and purification of the bioactive compounds and revealing their anticancer potential (Zeeshan et al., 2018).

Currently, therapeutic options for liver cancer include chemo-preventive therapy, radiotherapy, and surgery. Nevertheless, no effective and specific anti-liver cancer medication is available without hazardous effects. The study was planned to profile the phytoconstituents and investigate the therapeutic potential of *C. angustifolia* extracts against the liver cancer (HepG2) cell line.

Materials and Methods

Collection and identification of the plant

Leaves of the medicinal plant species, *Cassia angustifolia,* were collected in the summer from district Lahore and identified by a taxonomist (Prof. Zaheeruddin Khan) at the GC University, Lahore, Pakistan, under herbarium No. GC. Herb. Bot. 3908.

Preparation of plant leaf extracts

The leaves of *C. angustifolia* were dried for seven days in shade and then ground to powder form in a laboratory grinder (Thomas Scientific). The ground leaves (300 g) were soaked in 1 litre of ethanol (90%) and *n*-hexane (95%) at 37 °C for two weeks. Filtration was done using a filter paper (Whatman No. 1) and collected in reagent bottles. The filtrates from both extracts were concentrated using a rotary evaporator (Heidolph, Germany) at 40 ^{o}C. The extracts were dried further by lyophilization (freeze-

drying) and stored for further analysis (Kalsoom et al., 2022).

Yield of plant extracts

The yield of dried extracts was calculated using the following equation:

$$\text{Percent yield of extract (g)} = W1 / W2 \times 100$$

W1 represents the plant extract weight, and W2 represents the powdered material weight (Tulashie et al., 2021).

Characterization of plant extracts

The lyophilized ethanol (ECA) and *n*-hexane (HCA) extracts of *C. angustifolia* were sent to the International Center of Chemical and Biological Sciences (ICCBS) HEJ, University of Karachi, Karachi, Pakistan, for gas chromatography-mass spectrometric (GC-MS) analysis to profile phytocompounds (Painuli et al., 2016). The instrument, Agilent Technologies (7890A) GC-MS triple quad system with EI and CI ion source, was used for phytochemical analysis. Helium (99.99%) was used as a transporter gas at the rate of 1 mL per minute. The injector was run at 250 °C, while the oven operated at a holding time of 60 °C, followed by 10 °C per minute to 310 °C per 4 minutes. The National Institute of Standards and Technology (NIST) database, which contains over 62,000 patterns, was used to identify by comparing the spectra of known and unknown compounds. The peak area expression of the "TIC" (total ionic chromatogram) was used to measure the relative percentage levels of each component.

Cancer cell cultivation

Liver cancer (HepG2) and normal (HEK-293T) cell lines were arranged from the Cell-Bank (IMBB/CRiMM), The University of Lahore, Pakistan. Heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/mL Penicillin-Streptomycin (Caisson), and Dulbecco's Modified Eagle's Medium (DMEM) (Caisson) were used to grow cancer cells. On the other hand, Minimum Essential Media (MEM) (Gibco) was used for the normal human kidney cells (HEK-293T) with FBS (15%) and incubated at 37 °C with 5% CO_2. Slight modifications were made to the passage procedure after the cells reached 80% confluent. After removing the medium, PBS was used to wash the cells (Inovatiqa). To carry out trypsinization, trypsin (3 mL) (Gibco) was added till the adherent cells dislodged from the surface of the flask. Complete media (5 mL) was added to halt the reaction, and centrifugation was carried out at 1500 rpm for 5 minutes. As a part of the passage procedure, every 3 mL of cell suspension was transferred to a flask measuring T75 cm^2 (Masuku and Lebelo, 2019).

Cytotoxicity analysis of *C. angustifolia* extracts by MTT Assay

The *C. angustifolia* leaf extracts were evaluated for *in vitro* cytotoxicity on HepG2 and HEK-293T cell lines. Trypsin was used to detach the cells and then centrifuged. The cells were resuspended in the FBS medium, calculated using a haemocytometer, placed in 96-well microtitre plates, and incubated for 24 hours at 37 °C with 5% CO_2. The MTT assay was carried out using the method reported by Kalsoom et al. (2022). The stock solutions of ECA and HCA extracts were prepared using DMSO (Invitrogen Inc., USA) at a concentration of 160 mg/mL. A 96-well microtitre plate with 5×10^4 cells per well was seeded. The cells were then administered to 400, 200, 100, 50, and 10 µg/mL concentrations. The final stage was to incubate at the previously specified temperature with 5% CO_2. For HEK-293T cells, the same protocol was repeated using the MEM medium and 15% FBS. The cancer HepG2 and noncancer HEK-293T cells were used to evaluate the toxic properties of leaf extracts. Cisplatin drug (10 µg/mL) (Receipt No, 651278) and DMSO (0.1%) were used as positive and negative controls, respectively. The cells cultivated and treated with plain DMEM media containing 2% FBS were used to compare before and after treatment effects. The medium was aspirated, and a microscope was used to determine the level of cellular proliferation. The MTT reagent (20 µL) (Invitrogen Inc., USA) was added to the wells and then incubated for another two hours. After removing the supernatant, DMSO (150 µL) was used, and the formazan crystals were broken down by shaking the plates on a plate shaker. After incubation for 15 minutes, the absorbance spectra were measured at 570 nm using a spectrophotometer (BIO-RAD). For every sample, the reactions were carried out in triplicate. IC_{50} concentrations were calculated by applying the linear regression method (Kalsoom et al., 2022).

Cell Viability/Adhesion assay

In 9 mL of PBS, 0.1% WV of crystal violet (Sigma-Aldrich) was added to make a working solution. The cell viability (CV) assay was carried out adopting the protocol reported by Nawaz et al. (2021) with minor modifications. In a 96-well microtitre plate, HepG2 and HEK-293T cells (5×0^4 cells/well) were added in a

complete media (200 µL). The incubation for 72 h was spent at the predetermined temperature of 37 °C with humidity and 5% CO_2. For 10 minutes, the cells were fixed with 70% ethanol. After that, the CV solution (100 µL) was used to stain the cells and allowed to stay for 30 min. PBS was used to clear the cells from debris, and triton X-100 solution (200 µL) (Sigma-Aldrich CAS#9036-195) was added to remove the cellular stains. After 30 minutes of incubation at room temperature, the optical densities (OD) of the samples were measured at 570 nm using a spectrophotometer.

Percentage calculation of cell viability

The mathematical formula (equation) was used to determine the percentage of viable cells (Larsson et al., 2020).

$$\text{Percent Cell viability} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of control}} * 100$$

Morphological examination

The morphological variations in HepG2 and HEK-293T cells were observed and compared to the control group to examine the impact of plant extracts on cancer and non-cancerous cell proliferation using a phase contrast microscope and Floid Cell Imaging Station.

Data analysis

The experiments were performed in triplicates, and the data were presented as mean ± SD. A one-way analysis of variance (ANOVA), Graph Pad Prism 8.0 and Tukey's test were used to analyze the interaction of three variables. The linear regression approach was used to determine IC_{50} values. A *P*-value of < 0.05 with a 95% confidence interval (CI) was considered statistically significant (Kalsoom et al., 2022).

Results

The extract yield is the amount of extract obtained from the amount of total raw plant material used. The maximum percent yield obtained from the ECA and HCA extracts was 16.38% and 15.64%, respectively.

Phytochemical analysis of *C. angustifolia* extracts by GC-MS

The ECA and HCA extracts demonstrated the existence of various bioactive phytocompounds at various retention times through GC-MS. A total of 26 and 29 compounds were profiled in the ECA and HCA extracts. The identified phytocompounds are listed in **Table 1**, along with their molecular formulae (MF), retention times (RT), molecule weights (MW) and area sum percentages.

Cytotoxic effect of *C. angustifolia* extracts on HepG2 and HEK-293T cell lines

In the MTT assay, increasing concentrations (10, 50, 100, 200, and 400 µg/mL) of the ECA and HCA extracts were used to evaluate the cytotoxicity levels on the HepG2 and HEK-293T cell lines (**Figure 1**). Plant extracts exhibited a concentration-dependent decrease in cell growth at 72 h with IC_{50}=62 µg/mL for ECA and IC_{50}=67 µg/mL for HCA extracts on the liver cancer (HepG2) cell line. IC_{50} values reveal the better antiproliferative effects of ECA compared to those of HCA. However, the HCA extract showed less toxicity toward the liver cancer cells. The higher concentrations (200 and 400 µg/mL) of the ECA and HCA extracts showed better cytotoxicity when compared with the negative control (DMSO) and untreated (UT) cells. Both extracts, ECA (IC_{50}=245 µg/mL) and HCA (IC_{50}=482 µg/mL), showed less cytotoxicity on the HEK-293T cells. Cisplatin, on the other hand, always had a severe cytotoxic response not only toward cancer but also to healthy cells by restricting the growth, proliferation, and adhesion capacity.

Cell viability analysis of *C. angustifolia* extracts on HepG2 and HEK-293T cell lines

The crystal violet method calculated the percent cell viability of the HepG2 and HEK-293T cells treated with the ECA and HCA extracts. Various concentrations (10, 50, 100, 200, and 400 µg/mL) of both extracts showed percent viabilities of 98%, 79%, 45%, 30%, and 25% for the ECA and 95%, 68%, 55%, 40%, and 31% HCA extract, respectively (**Figure 2**). The results were comparatively analyzed with DMSO (0.1%) and UT (100%), which showed a significant decrease in cell number, revealing that both extracts had cytotoxic effects on the selected cell lines according to their concentrations. However, the results demonstrated the negligible effect of the ECA (95%, 76%, 74%, 70%, and 64%) and HCA extracts (92%, 85%, 77%, 78%, and 74%) on the viabilities of the HEK-293T cells. In comparison, cisplatin showed an inhibition of < 40% in cancer cells and < 38% in normal cells. The difference in inhibition was noticeable at all concentrations. Our findings revealed a statistically significant correlation (R^2 =0.9616, 0.9444, 0.9954, and 0.9891, *P*-value < 0.05) for both ECA and HCL extracts.

Table 1. GC-MS identified phytocompounds from *C. angustifolia* extracts

Compounds name	Molecular formula	RT	ECA extract		HCA extract	
			MW	Area %	MW	Area %
Isoamyl acetate	$C_7H_{14}O_2$	7.7	130	0.29	130	0.71
2,3-dihydroxypropyl acetate	$C_5H_{10}O_4$	10.0	-	-	134	0.27
2,7,10-trimethyldodecane	$C_{15}H_{32}$	10.6	212	0.32	-	-
Octadecane	$C_{18}H_{38}$	13.5	254	0.66	-	-
Dihydroactinidiolide	$C_{11}H_{16}O_2$	14.0	-	-	180	0.28
Fumaric acid, ethyl 2-methylallyl ester	$C_{10}H_{14}O_4$	14.4	-	-	198	0.19
Ethyl alpha-D-glucopyranoside	$C_8H_{16}O_6$	14.9	208	1.73	-	-
2,6,10-trimethyltetradecane	$C_{17}H_{36}$	15.9	240	0.68	-	-
Phenol, 2-methyl-5-(1,2,2-trimethylcyclopentyl)-, (S)-	$C_{15}H_{22}O$	16.4	-	-	218	0.18
Myristic acid	$C_{14}H_{28}O_2$	16.5	-	-	228	0.17
1,5,9,9-tetramethyl-spiro[3.5]nonan-5-ol	$C_{13}H_{24}O$	16.6	-	-	196	0.15
3,7,11,15-tetramethyl-2-hexadecen-1-OL	$C_{20}H_{40}O$	17.3	296	0.36	278	0.62
7,9-di-tert-butyl-1-oxaspiro [4.5]deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	18.4	276	0.95	-	-
Palmitic acid	$C_{16}H_{32}O_2$	18.9	256	6.03	-	-
n-hexadecanoic acid	$C_{16}H_{32}O_2$	19.0	-	-	256	8.97
Ethyl palmitate	$C_{18}H_{36}O_2$	19.4	284	2.58	284	0.61
Phytol, isophytol	$C_{20}H_{40}O$	21.7	296	6.04	296	2.22
Linolenic acid	$C_{18}H_{30}O_2$	22.3	278	8.39	278	18.06
(9Z,12Z)-ethyl octadeca-9,12-dienoate	$C_{20}H_{36}O_2$	22.9	308	1.45	-	-
Ethyl 9,12,15-octadecatrienoate	$C_{20}H_{34}O_2$	23.1	306	3.15	-	-
Phytol	$C_{20}H_{40}O$	24.6	-	-	296	3.79
1-monolinolein	$C_{21}H_{38}O_4$	42.8	354	0.45	-	-
1-monolinolenoyl-rac-glycerol	$C_{21}H_{36}O_4$	42.9	352	1.32	-	-
Heptacosane	$C_{27}H_{56}$	43.1	380	1.59	380	4.39
(E,E,E,E)-squalene	$C_{30}H_{50}$	44.6	410	19.26	410	28.29
Heptacosane	$C_{30}H_{50}$	45.3	410	1.02	-	-
Nonacosane	$C_{29}H_{60}$	45.3	-	-	408	2.14
1-heptatriacotanol	$C_{37}H_{76}O$	45.6	536	1.88	-	-
1,6,10,14,18,22-tetracosahexaen -3-ol, 2,6,10,15, 19,23-hexamethyl -, (all-E)-	$C_{30}H_{50}O$	45.6	-	-	426	1.39
2,2,4-trimethyl-3-[(3E,7E,11E)-3, ,8,12,16-tetramethylheptadeca-3, 7,11,15-tetraenyl]cyclohexan-1-ol	$C_{30}H_{52}O$	45.7	-	-	428	0.4
2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-,(all-E)-	$C_{30}H_{50}O$	46.2	-	-	426	0.38
1,6,10,14,18,22-tetracosahexaen -3-ol ,2,6,10,15,19,23-hexamethyl-,(all-E)-	$C_{30}H_{50}O$	46.3	-	-	426	0.39
24,25-dihydroxycholecalciferol	$C_{37}H_{76}O$	46.6	416	0.4	-	-
Gamma-tocopherol	$C_{28}H_{48}O_2$	46.6	-	-	416	0.66
hentriacontane	$C_{31}H_{64}$	47.0	-	-	436	1.56
Octacosyl acetate	$C_{30}H_{60}O_2$	47.0	452	2.21	-	-
Vitamin E	$C_{29}H_{50}O_2$	47.3	430	11.15	430	16.88
Stigmasterol	$C_{29}H_{48}O$	48.4	412	1.52	412	0.8
Clionasterol	$C_{29}H_{50}O$	48.9	414	1.62	414	0.7
Stigmasta-5,22-dien-3-ol acetate	$C_{31}H_{50}O_2$	49.7	-	-	454	0.42
Triacontyl acetate	$C_{32}H_{64}O_2$	49.9	-	-	480	0.65
Silane, dimethyl(docosyloxy)butoxy-	$C_{28}H_{60}O_2Si$	50.1	456	15.2	-	-
Stigmastan-3,5-diene	$C_{29}H_{48}$	50.4	-	-	396	0.7
Ethyl iso-allocholate	$C_{26}H_{44}O_5$	52.6	-	-	436	0.57
Androst-7-ene-6,17-dione, 2,3,14-trihydroxy-, (2beta, 3beta, 5alpha)-	$C_{19}H_{26}O_5$	52.6	334	9.75	-	-

ECA: Ethanol extract of *C. angustifolia;* HCA: hexane extract of *C. angustifolia;* RT: retention time; MW: molecular weight

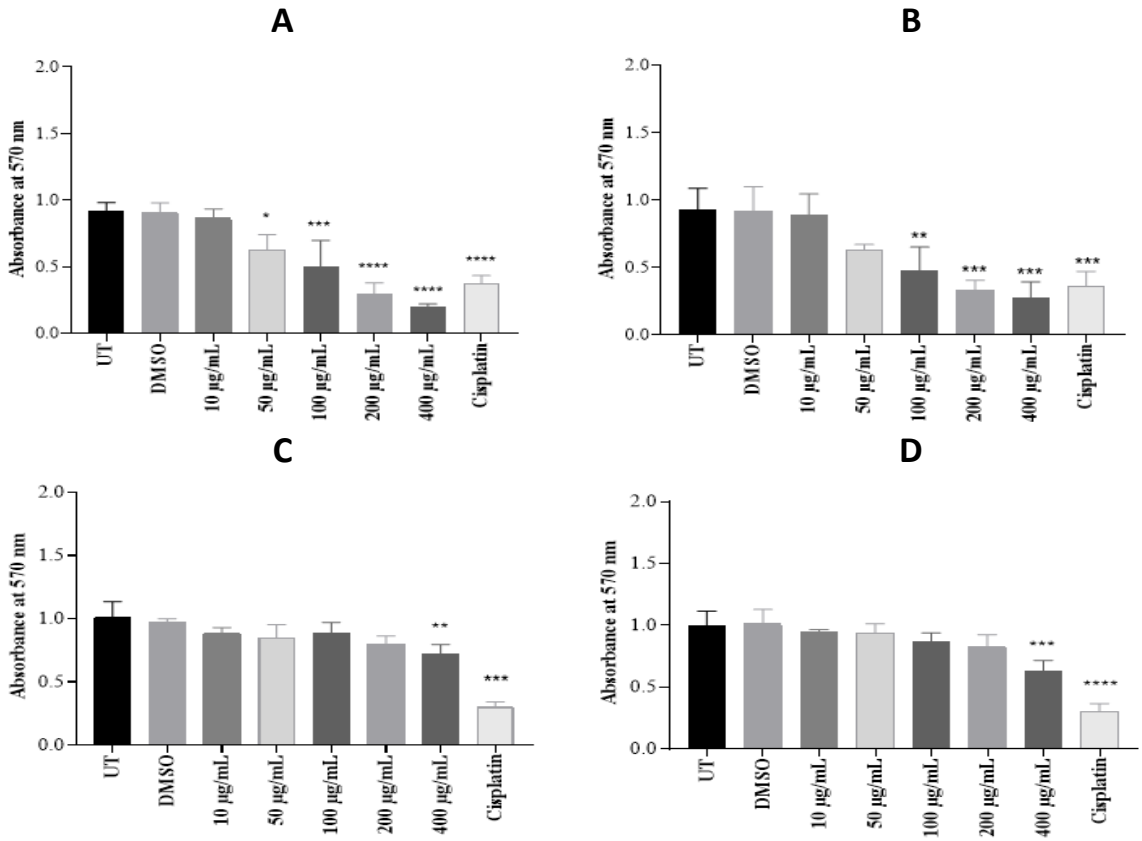

Figure 1. Graphical representation of the antiproliferative effect of *C. angustifolia* extracts on HepG2 and HEK-293T cells (A) Inhibitory effects of ECA extract on HepG2 cell line. The MTT assay showed increased mortality of cancer cells at all concentrations; (B) Dose-dependent antiproliferative effect of HCA concentrations represents a significant decrease in HepG2 cells; (C) HEK-293T cell line showed minimal or no response with ECA extract exposure; (D) HCA effects on healthy cell line also showed less or no activity. Cisplatin response revealed more encouraging effects against normal and cancer cell lines. The data is represented as the mean ± SD of each group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate statistically significant differences between the control and treated cells. UT: Untreated; DMSO: Dimethyl sulfoxide; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); ECA: Ethanol extract of *C. angustifolia*; HCA: *n*-hexane extract of *C. angustifolia*

Morphological examination of HepG2 and HEK-293T cells treated with ethanol extract of *C. angustifolia*

The morphological changes in the cancer HepG2 and noncancer HEK-293T cells were observed using an imaging station and an inverted phase contrast microscope after 72 h of treatment with the ECA extract (**Figure 3**). The cells were seeded in a 96-well plate and treated with 10, 50, 100, 200, and 400 µg/mL concentrations of the ECA extract. The proliferation of the liver cancer cells was significantly reduced at 100, 200, and 400 µg/mL concentrations of the ECA extract. However, a distinctive change in the growth of cells was noticed after 72 h of incubation. This observation supports the conclusion that the ECA extract has potential antiproliferative effects against the HepG2 cells at higher concentrations. No significant changes in the HEK-293T cells were observed at different doses (10, 50, 100, 200, and 400 µg/mL) of the extract. The cisplatin (positive control) showed significant morphological effects on both cancer and noncancer cell lines.

Morphological examination of HepG2 and HEK-293T cells treated with *n*-hexane extract of *C. angustifolia*

Changes in the morphology of liver cancer (HepG2) cells were noticed after the treatment of the HCA extract in a dose-dependent manner (10, 50, 100, 200, and 400 µg/mL) compared to the negative control (DMSO). Higher concentrations (200 and 400 µg/mL) of the HCA extract showed significant results such as shrinkage of cells, and cytoplasmic condensation (**Figure 4**). The HEK-293T cells did not show marked damage to the cells by the HCA extract, however, cisplatin induced cell rounding and growth restriction-like changes in the cancer cells as well as in the healthy ones.

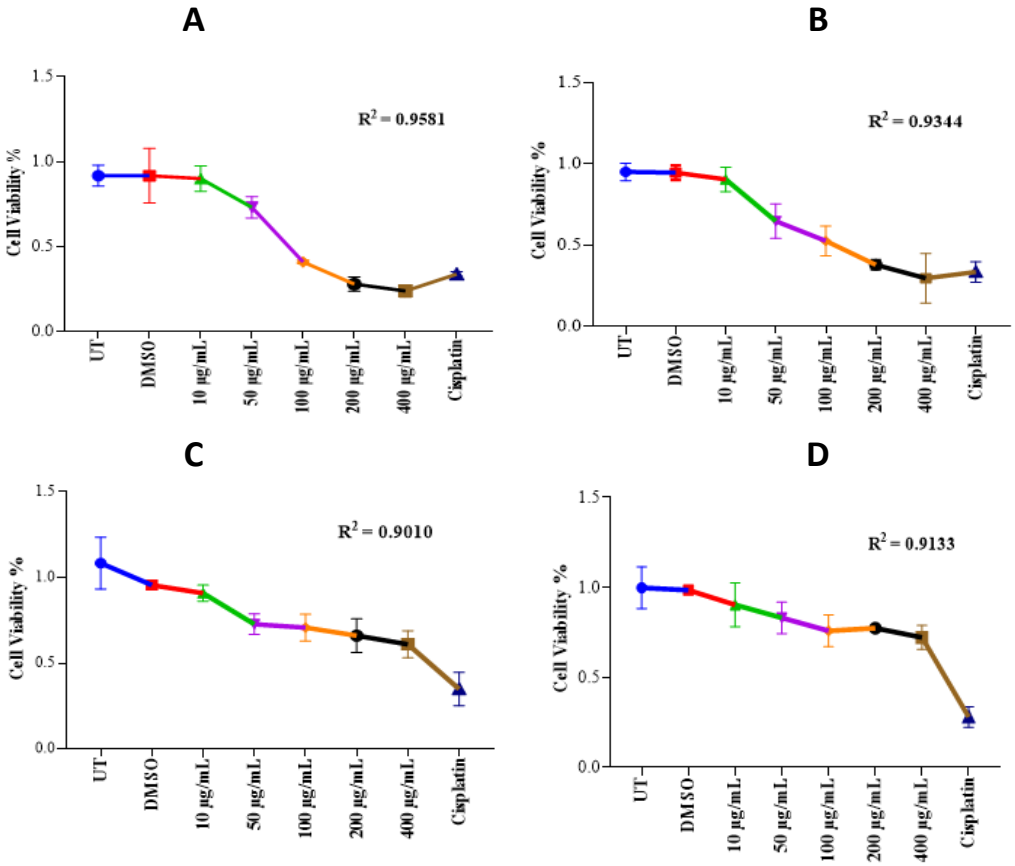

Figure 2: Cell viability analysis of *C. angustifolia* extracts on HepG2 and HEK-293T cells; (A) Cell viability percentage was evaluated by the crystal violet staining method represented as a trend-line slope. ECA extract concentrations exhibited a promising reduction in the percent viability of HepG2 cells; (B) The HCA extract concentrations exhibited a strong dose-dependent decrease in cancer HepG2 cells; (C) The effect of ECA extract on the HEK-293T cell line showed a slight fall in percent viability; (D) the HCA extract did not show severe toxicity on normal cells. All treatments differed significantly from untreated and control groups and are expressed as *P < 0.05, **P < 0.01, ***P < 0.001. UT: Untreated; DMSO: Dimethyl sulfoxide; ECA: Ethanol extract of *C. angustifolia*; HCA: *n*-hexane extract of *C. angustifolia*

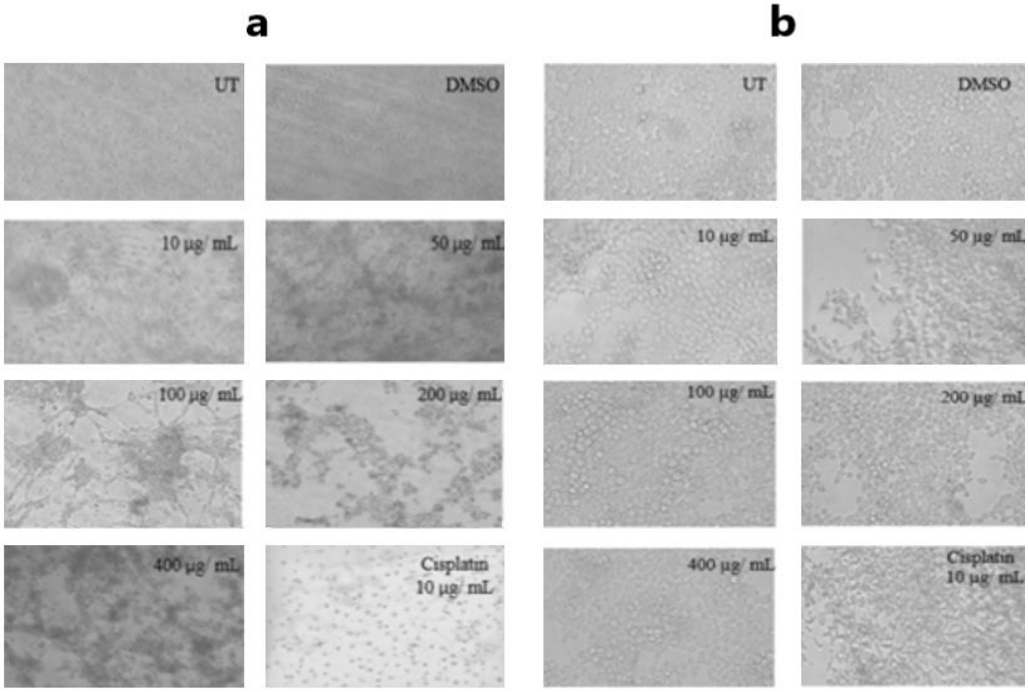

Figure 3. Morphological representation of HepG2 and HEK-293T cells exposed to different concentrations of *C. angustifolia* ethanol extract; (a) Morphological characteristics of HepG2 cells treated with 10, 50, 100, 200, and 400 µg/mL concentrations of the ECA extract for 72 h; (b) HEK-293T cells represent normal morphology. Cisplatin revealed strong effects on both cell lines. UT: Untreated; DMSO: Dimethyl sulfoxide; ECA: Ethanol extract of *C. angustifolia*

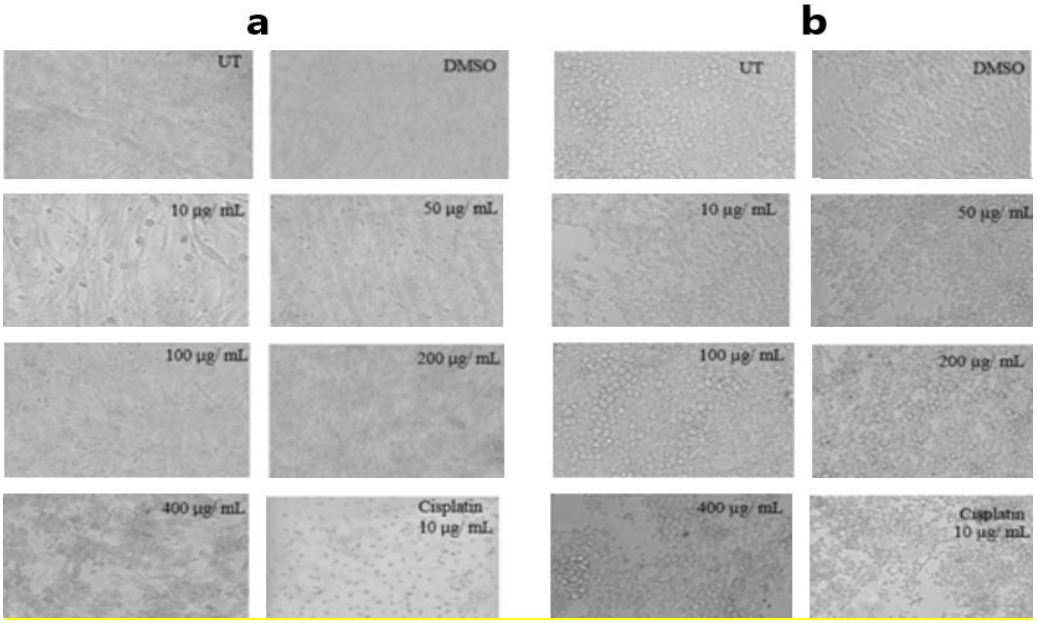

Figure 4: Morphological representation of HepG2 and HEK-293T cells exposed to different concentrations of *C. angustifolia* *n*-hexane extract; (a) Effect of HCA extract at 10, 50, 100, 200, and 400 µg/mL concentrations on the HepG2 cells for 72 h; (b) The HEK-293T cells represent normal morphology at all concentrations. Cisplatin had a strong effect on both cell lines. UT: Untreated; DMSO: Dimethyl sulfoxide; HCA: *n*-hexane extract of *C. angustifolia*

Discussion

The current study investigated the extracts of *Cassia angustifolia* that possess a variety of phytoconstituents, including alkaloids, anthocyanins, anthraquinones, carbohydrates, cardiac glycosides, diterpenes, flavonoids, leucoanthocyanins, phenols, phytosterols, saponins, tannins, and terpenoids; all these have been documented to possess anticancer, antibacterial, antioxidant and other bioactivities (**Table 1**). Based on the percent composition, the notable compounds in the ECA extract were squalene (19.26%), silane, dimethyl (docosyloxy)butoxy (15.2%), vitamin E (11.15%), androst-7-ene-6,17-dione, 2,3,14-trihydroxy-, (2beta, 3beta,5alpha) (9.75%), linolenic acid (8.39%), phytol (6.04%), and palmitic acid (6.03%). Squalene, a triterpene, is a precursor of non-steroidal cholesterol. It is synthesized as acetyl-CoA, a metabolic intermediate reported to have anti-atherosclerotic, anticancer, and antioxidant properties (Purkiewicz et al., 2022). Linoleic acid is an essential polyunsaturated omega-3 fatty acid and an intermediary in the biosynthesis of hormone-like eicosanoids, and it controls immune response and inflammation (Shim et al., 2014). Phytol is a diterpene acyclic alcohol that induces acidic vesicle formation, protein kinase B (Akt), p70S6K activation, and the mTOR (mechanistic target of rapamycin) pathway to be downregulated in human gastric adenocarcinoma (AGS) cells (Islam et al., 2018). Palmitic acid (6.03%) acts as 5-alpha-reductase inhibitor in developing prostate cancer (CaP). It also demonstrates antimicrobial, anti-fibrinolytic, anti-inflammatory, antioxidant, hemolytic, metabolic, and anticancer activities in several types of cancers (Zhu et al., 2021). The presence of GC-MS profiled biologically active phytochemicals in the ECA plant extract are known to be suitable for addressing different ailments (Gomathi et al., 2015). On the other hand, as in the ECA extract, the HCA extract also possessed large amounts of chief biologically active chemicals, including squalene (28.29%), linolenic acid (18.06%), vitamin E (16.88%), heptacosane (4.39%), and phytol (3.79%). Out of 45, 10 phytocompounds (Isoamyl acetate, 3,7,11,15-tetramethyl-2-hexadecen-1-OL, ethyl palmitate, phytol, linolenic acid, heptacosane, (E,E,E,E)-squalene, vitamin E, stigmasterol, and clionesterol (**Table 1**).

The results showed that the ECA (IC50=62 µg/mL) and HCA extracts (IC50=67 µg/mL) displayed significant antiproliferative activity on HepG2 cells, while HEK-293T (ECA: IC50=245 µg/mL; HCA: IC50=482 µg/mL) has shown minimal cytotoxicity against higher concentrations (**Figure 1**). Ahmed et al. (2016) used *Cassia angustifolia* extracts (ethanol and methanol) and obtained the IC50 and cell viability at 100, 150, 200, and 250 µg/µL against cancer (MCF-7, HeLa, and HepG2) and normal (HCEC) cell lines using the MTT calorimetric assay. Ethanol concentrations showed 28% viability with IC50 =7.28 µg/µL in the HepG2 cells. Methanol exhibited 33% viability with IC50 = 5.45 µg/µL in HeLa cells and 43% with IC50 = 4 µg/µL in MCF-7 cells. Noncancer (HCEC) cells exhibited 100% viability toward both extracts (Ahmed et al., 2016). The authors also showed that the alkaloids in the *C. angustifolia* extracts exhibited significant antiproliferative activity on HepG2 cells, arbitrated by an extracellular signal-regulated kinase (ERK)

inhibition, a member of the family of mitogen-activated protein kinases involved in cell growth and regulation, and downregulation of cyclin D1. Thus, it can be presumed that Senna is a potent antitumor agent or, at least, can be used as an adjuvant in treating colorectal and liver cancer (Al-Dabbagh et al., 2018). Our study showed significant antiproliferative activity for HepG2 compared to the HEK-293T cells at all tested concentrations. The MTT assay authorizes to explore the cytotoxic properties of the *C. angustifolia* extract concentrations against the cancer HepG2 cell line compared to the normal HEK-293T. Therefore, the substantial cytotoxic effect of the ECA and HCA extracts can be attributed to the occurrence of valuable anticancer phytochemicals.

A cytotoxic potential of the ECA and HCA extract concentrations was employed to assess the viability of the HepG2 and HEK-293T cells by the crystal violet assay (**Figure 2**). There is an inverse correlation between the ECA extract and the cell viability of cancer cells. It was also noticed that the cancer cell viability reduced with increased concentrations of the extracts after 72 h of incubation. *Cassia angustifolia* extracts were toxic to the HepG2 cells where the percent cell viability was reduced to 98%, 79%, 45%, 30%, and 25% for ECA and 95%, 68%, 55%, 40%, and 31% for the HCA extract. The literature reported the maximum percentage of cell viability (92%) in *C. angustifolia* ethanolic leaf extract in the HepG2 cells at 200 µg/mL (Ahmed et al., 2016). However, the liver cancer cells have shown cell viability of 30% for the ECA and 40% for the HCA extracts at 200 µg/mL, indicating that our results are much better than the previously reported against hepatocellular carcinoma. The HEK-293T cells demonstrated negligible effects of the ECA and HCA extracts at all concentrations revealing their specific action towards cancer cells only, and safe for normal cells. In contrast, cisplatin showed less than 40% in the cancer cells and 38% in the normal cells.

In this study, the ECA and HCA extracts exhibited various apoptotic characteristics, including apoptotic body formation, blabbing of the membrane, and shrinkage of cells. The results were comparatively analyzed with UT (100%), and DMSO-treated cells showed a significant decrease in cell number (**Figure 3**; **Figure 4**). According to the results, the viability of the cells was reduced significantly when the extract concentrations increased. Furthermore, the apoptotic morphological changes became more evident with increasing concentrations of leaf extracts.

Conclusion

In the current research, both ethanol and hexane extracts of *C. angustifolia* leaves exhibited a strong cytotoxic potential on liver cancer (HepG2) and minimal effects on healthy (HEK-293T) cells. The GC-MS analysis proved the presence of bioactive phytocompounds, responsible for the anticancer action of *C. angustifolia* extracts. Further research is necessary to isolate the active compounds and reveal their underlying mechanisms to cause cytotoxicity in cancer cells.

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As per clarification of corresponding author, this research does not directly involve human or animal subjects. Therefore, a certificate of ethical approval is not applicable.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discorded to minimize any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

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