Investigation of the Cytotoxic Activity of Ethanolic Banaba (Lagerstroemia speciosa) Leaf Extract Through the Intrinsic Mitochondrial Apoptotic Pathway in HepG2 Cells

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide. Despite advances in treatment, conventional chemotherapeutic agents are associated with significant toxicity and limited efficacy. Plant-derived bioactive compounds have emerged as promising alternatives due to their selective anticancer properties. *Lagerstroemia speciosa* (Banaba) contains several phytochemicals, including corosolic acid, berberine, and gallic acid, which possess potential anticancer activity. **Aim:** To investigate the cytotoxic and apoptosis-inducing effects of ethanolic Banaba (*Lagerstroemia speciosa*) leaf extract (EBLE) against HepG2 hepatocellular carcinoma cells through the intrinsic mitochondrial apoptotic pathway. **Materials and Methods:** HepG2 cells were treated with varying concentrations of EBLE (25–150 µg/mL). Cytotoxicity was assessed using the MTT assay. Oxidative stress markers, including lipid peroxidation (LPO), superoxide dismutase (SOD), and reduced glutathione (GSH), were estimated. Apoptosis was evaluated by acridine orange/ethidium bromide (AO/EB) dual staining and JC-1 mitochondrial membrane potential assay. Quantitative real-time PCR was performed to analyze the expression of apoptosis-related genes, including Akt, Bax, Bcl-2, cytochrome c, Apaf-1, caspase-9, and caspase-3. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's multiple comparison test, with $p<0.05$ considered statistically significant. **Results:** HPTLC analysis confirmed the presence of berberine and gallic acid in EBLE. The extract produced concentration-dependent cytotoxicity in HepG2 cells, with an IC_{50} of 150 µg/mL ($p<0.001$). EBLE significantly increased LPO and SOD levels while altering intracellular GSH concentrations, indicating oxidative stress. AO/EB staining demonstrated a marked increase in apoptotic cells, whereas JC-1 staining revealed significant loss of mitochondrial membrane potential. Gene expression analysis showed downregulation of Akt and Bcl-2, along with significant upregulation of Bax, cytochrome c, Apaf-1, caspase-9, and caspase-3, confirming activation of the intrinsic mitochondrial apoptotic pathway. **Conclusion:** Ethanolic Banaba leaf extract exhibited significant cytotoxic activity against HepG2 cells by inducing oxidative stress-mediated mitochondrial apoptosis. The modulation of apoptosis-related genes and activation of the intrinsic apoptotic pathway suggest that EBLE may serve as a promising natural therapeutic candidate for hepatocellular carcinoma. Further in vivo and clinical studies are warranted to establish its therapeutic efficacy and safety.

KEYWORDS: Hepatocellular carcinoma; *Lagerstroemia speciosa*; Mitochondrial apoptosis.

How to cite this article: Jagitharam RR, Singh TR. Investigation of the Cytotoxic Activity of Ethanolic Banaba (Lagerstroemia speciosa) Leaf Extract Through the Intrinsic Mitochondrial Apoptotic Pathway in HepG2 Cells. Int J Drug Deliv Technol. 2026;16(68s):739-747. DOI: 10.25258/ijddt.16.68s.91

Introduction

Hepatocellular carcinoma (HCC) accounts for more than 90% of all cases of primary liver cancer and is responsible for significant morbidity and mortality worldwide.[1] The incidence of HCC has been increasing every year and the World Health Organization considers HCC as the second leading cause of cancer.[2] In addition, HCC is responsible for 9.1% of all cancer deaths due to poor prognosis.[3] Hepatitis B and C virus infections, ingestion of aflatoxin B1 contaminated food, and chronic ethanol consumption are some of the potential risk factors for HCC development.[4]

Current treatment modalities for HCC involve chemotherapy with cytotoxic drugs, targeted drug therapies with different growth factor inhibitors, tyrosine kinase inhibitors, liver transplantation, and so on.[5] Targeted therapies have proved their efficacy in blocking angiogenesis factor and are considered to be the choice of treatment in advance cases of HCC.[6] In a recent study, sorafenib and apatinib, tyrosine kinase inhibitors were shown to be effective in patients with liver can- cer. However, all these treatment modalities are associated with some adverse effects.[5,7] Moreover, liver cancer is less sensitive to chemo- therapy, and cytotoxic anticancer drugs are commonly employed to control metastasis and proliferation of cancer cells. They often cause toxicity to non-cancer cells. Hence, there is an unmet need for the development of novel chemotherapeutic agents

with fewer or no tox- icity profile.

Phyto-constituents derived from various plant products are used in treatment of wide range of cancers as their therapeutic role is well studied.[8] Many herbal compounds have been studied for their curative properties, and some of them have been proven to be effec- tive against HCC by targeting various drivers of HCC.[8,9] Anticancer effect of herbal medicines are mainly mediated through immuno- modulation, cell cycle arresting, and apoptosis inducing potential in cancer cells.[10]

Lagerstroemia speciosa Pers. (Lythraceae) commonly known as Banaba (Pride of India) has been used in Indian traditional medicine for the treatment of a variety of diseases which includes diabetes and obesity.[11] *L. speciosa* consists of several phytoconstituents like glyco- sides, flavones, corosolic acid, ellagic acids, triterpenes, tannins, and so on which are reported to present in leaves, stem, flowers, fruit, bark, and roots.[11,12] Among them, corosolic acid, a triterpenoid pre- sent in banaba, has proven to have more therapeutic role in the treatment of variety of diseases.[13] Banaba as an anti-inflammatory, anti-diarrhoeal, and anti-gout agent is well established in different in vivo models.[11] In a previous study, we have explored the cell cycle arresting potential of ethanolic Banaba leaf extract (EBLE) in liver can- cer cells.[14] In the present study, apoptosis-inducing potential of EBLE was investigated against HepG2 cell lines.

AIMS AND OBJECTIVES

1.To investigate cytotoxic potentials of EBLE in HepG2 cells.

2.To investigate apoptosis inducing potential of EBLE against HepG2 cell lines.

3.To analyze EBLE -induced changes related to apoptosis via intrinsic mitochondrial pathway in HepG 2 cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's minimum essential low glucose medium (DMEM), dimethyl sulfoxide (DMSO), penicillin, streptomycin, tryspin-EDTA, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS) were obtained from GIBCO BRL (Gaithersburg, MD). Forward and reverse primers of β-actin, Bcl-2, Bax, cytochrome C, caspase 3 and 9, Apaf-1, and Akt were purchased from Shrimpex. All other chemicals were of analytical grade.

Plant material and extraction

Ethanolic leaf extract of *Lagerstroemia speciosa* (L.) Pers. was pur- chased from Quimico (Batch no. KAN/BE/1801009) and Herbal Extract Manufacturer, Bengaluru, India. According to the manufac- turer's certificate of analysis, EBLE contained 20% corosolic acid which was analyzed and proved by high-performance liquid chroma-tography assay.[14]

High-performance thin layer chromatography analysis

The high-performance thin layer chromatography (HPTLC) was per- formed according to a previous method.[15] Briefly, EBLE (5 mg) was dissolved in 1 mL of chromatography grade methanol and used for sample application on pre-coated silica gel 60 GF_{254} (10 × 10 cm with 250 μM thickness) used as stationary phase. HPLC grade chloroform: methanol (9.5:0.5), isopropanol:formic acid:water (4.5:0.1:0.4), and chloroform:ethyl acetate:formic acid (2.5:2:0.8) were used as mobile phases for corosolic acid, berberine, and gallic acid, respectively. Pure compounds of the same was dissolved in methanol (50 μg/mL) and used as standards. Development was done in ascending mode using CAMAG Automatic TLC Sampler 4 (ATS4), which was programmed through winCATS software.

Cell culture

The HepG2 human HCC cell line was procured from NCCS, Pune, Maharashtra, India, with the passage number of 16. Cells were maintained in DMEM with low glucose supplemented with 10% FBS containing penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were cultured with 5% CO_2 at 37°C and were grown in 25-cm^2 culture flask.[4] After a few passages, the cells were seeded for experi- ments. The experiments were done at 70%-80% confluence. Upon reaching confluence, cells were detached using 0.25% Trypsin-EDTA. The EBLE was dissolved in 0.1% DMSO.

MTT assay

The cytotoxic evaluation of EBLE was done using the MTT assay.[16] Cells were seeded in 96-well plate at a concentration of 1×10^4 cells/ well and incubated for 24 hours. Then, existing medium was aspirated, and fresh medium (100 μL) containing EBLE each at different concen-trations (25, 50, 100, and 150 μg/mL) was incubated for 24 hours. At the end of the treatment period, media from control and EBLE treated cells were discarded, and 50 μL of MTT (0.5 mg/mL) was added to each well. Cells were then incubated for 4 hours at 37°C in CO_2 incu-bator. MTT was then discarded, and the colored formazan were dis- solved in 150 μL of DMSO. The purple-blue formazan formed was measured using a Perkin Elmer multimode Reader at 570 nm. The optical density of each sample was compared with control optical den- sity, and graphs were plotted. Based on the IC_{50} evaluation, the fol- lowing studies were carried out with 50, 100, and 150 μg/mL of EBLE concentrations for 24 hours. Silymarin (50 μg/mL) was used as posi-tive control in this study.

Estimation of oxidative stress markers

Briefly, cells were seeded in six-well plate and incubated with/without different concentrations of the EBLE (50, 100, and 150 µg/mL) and SIL (50 µg/mL), respectively, for 24 hours. Cells were collected, washed with cold PBS, and homogenized using RIPA buffer. Then, it was centrifuged at 1500 rpm for 5 minutes, and the supernatant was immedi- ately used for lipid peroxidation (LPO), superoxide dismutase (SOD), and glutathione (GSH) estimation. Total protein was estimated by the method of Lowry et al.[17] Estimation of lipid peroxidation involved heating of 0.2 mL of the EBLE and SIL-treated cell lysate supernatant with saline, butylated hydroxy toluene, and thiobarbituric acid reagent in a boiling water bath for 1 hour. Then, it was centrifuged at 2000 rpm for 10 minutes, and the precipitate obtained was removed. The absor- bance of the supernatant was determined at 532 nm using a spectrophotometer.[18] SOD activity was assayed by the method based on the inhibition of the formation of NADH-PMS-NBT complex.[19] Reduced GSH content was measured by the method based on the development of a yellow color, when 5, 5-dithiobis (2-nitrobenzoic acid) was added to compound containing sulfhydryl groups.[20]

Acridine orange/ethidium bromide (AO/EB dual staining)

Acridine orange/ethidium bromide (AO/EB) staining was carried out by the method of Kasibhatla et al.[21] At the end of the treatment, the culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then, equal volumes of cells from control and EBLE treatments were mixed with 100 µL of dye mixture (1:1) of AO/EB and viewed immediately under Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY). A minimum of 200 cells was counted in each sample at five different fields. The percentage of apoptotic cells was determined dividing total number of apoptotic cells with total number of cells counted.

Mitochondrial membrane potential (ΔΨm) by JC-1 staining assay

Briefly, 1×10^4 HepG2 cells were plated in a six-well plate and cul- tured overnight. Then, cells were treated with different concentra- tions of EBLE and SIL for 24 hours. Then, cells were washed with PBS and stained with JC-1 (5 mg/mL) for 20 minutes at 37°C in the dark. Then, ΔΨm depletion was observed under a Nikon Eclipse Ti fluores- cence microscope (Nikon Instruments Inc.).[22]

Gene expression analysis by real time PCR

Cells from control, EBLE, and SIL treatments were collected, and total RNA was extracted by Trizol reagent according to the manufacturer instructions. Isolated RNA was quantified using a Nanodrop, and cDNA was synthesised using a commercial kit (high cDNA, Applied Biosystem). Quantitative real-time PCR (qPCR) was performed in AbiPrism 7700 sequence detection system (Applied Biosystems). Gene-specific primers for Akt, Bax, Bcl-2, cytochrome c, caspase 3 and 9, and Apaf-1 were used. β-Actin was used as house keeping control. The change in fluorescence of SYBR Green dye in every cycle was monitored, and the threshold cycle (ct) above background for each reaction was calculated. Results are expressed as fold change in gene expression with respect to the controls.[23]

Statistical analysis

Data were expressed as mean ± SEM and analyzed by one-way ANOVA followed by Dunnett's test multiple comparison test to determine the significant differences between groups. $P < .05$ was consid- ered as significant (Graph Pad prism 7.0. CA).

RESULTS

Phytochemical analysis of EBLE

The presence of berberine and gallic acid in EBLE extract has been analyzed by the HPTLC method. Chromatograms show the presence of the above phyto-constituents in EBLE (Figure 1) with the Rf values of 0.24 vs 0.23 and 0.44 vs 0.39 of their corresponding standards, respectively, was detected in EBLE extract. The quantitative analysis showed the presence of 3.19 µg and 2.94 µg of berberine and gallic acid, respectively, per mg of EBLE extract.

EBLE treatments induced cytotoxicity in HepG2 cells

Figure 2A shows the morphology of HepG2 cells exposed to differ- ent concentrations of EBLE. HepG2 cells showed typical cancer cell morphology. HepG2 cells treated with EBLE for 24 hours caused a significant ($P < .001$) and concentration-dependent cytotoxicity. The maximum cytotoxic effect was observed with the highest con- centration of EBLE, that is, 150 µg/mL used in this study. The low- est concentrations of EBLE, that is, 25 and 50 µg/mL treatments did not affect the viability of HepG2 cells. SIL treatment produced a sig- nificant loss of viability when compared to untreated control. Inter- estingly, a high concentration of EBLE at 150 µg/mL treatment caused significant cytotoxicity similar to that of SIL treatment (Figure 2B). The IC_{50} of EBLE in HepG2 cells was found to be 150 µg/mL. Therefore, further studies were carried out with the concentration ranges below the IC_{50} of EBLE such as 50, 100, and 150 µg/mL.

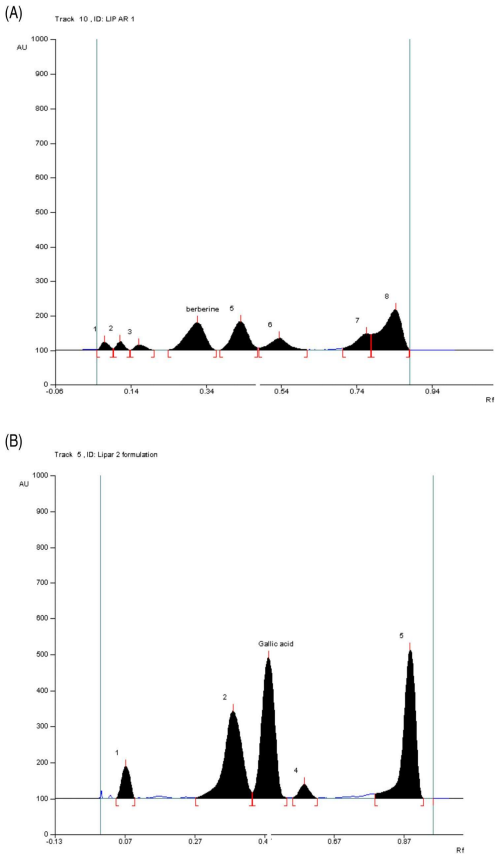


Figure 1: Phytochemical analysis of ethanolic banaba leaves extract (EBLE) by HPTLC. (A and B) Chromatograms showing the presence of berberin (with the Rf value of 0.24 vs standard Rf with 0.23) and gallic acid (with the Rf value of 0.44 vs standard Rf with 0.39), respectively.

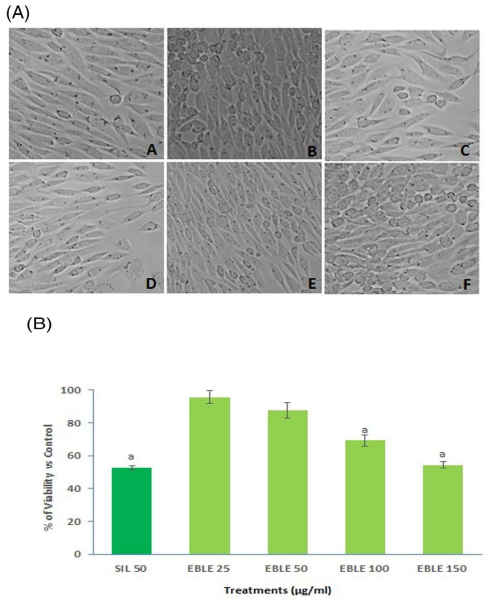


Figure 2. Cytotoxic effect of ethanolic banaba leaves extract (EBLE) in HepG2 cells. (A) Morphology of HepG2 cells exposed to various concentrations of EBLE (200×). A, control; B, Silymarin (SIL) 50 µg/mL; C-F, EBLE 25, 50, 100, 150 µg/mL, respectively. (B) Effect of EBLE on the viability of HepG2 cells. Data presented mean ± SEM, n = 3, aP < .001 vs control

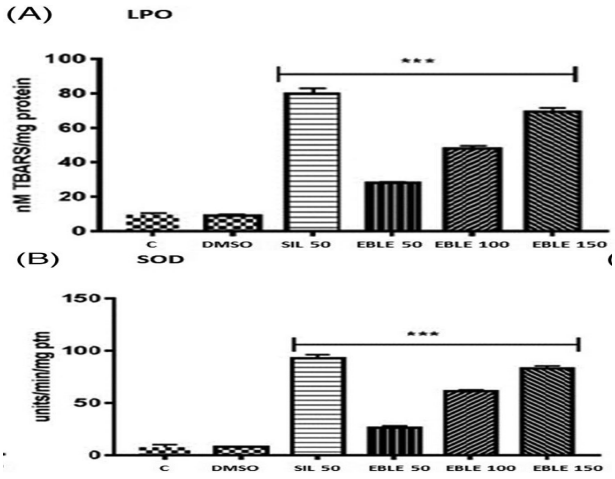
EBLE treatments induced changes in the status of LPO, GSH, and SOD activity in HepG2 cells

To ascertain whether the induction of oxidative stress was responsible for the loss of EBLE induced viability, we evaluated oxidative stress markers. Control and DMSO treated cells did not induce any significant change in the status of LPO, GSH, and SOD activity in HepG2 cells. However, SIL and EBLE treatments for 24 hours caused a significant (P < .001) and concentration-dependent elevation of LPO and SOD in HepG2 cells (Figure 3A and B). Furthermore, EBLE extract especially in higher concentrations, that is, 100 and 150 µg/mL induced SOD level highly significantly (P < .001). Low concentration of EBLE produced a Figure 3C).

EBLE treatments induced apoptosis-related morphological changes in HepG2 cells

To find out whether EBLE-treatments induced oxidative stress as a cause of apoptosis related morphological changes, we performed dual staining with AO/EB. Control and DMSO-treated cells appeared as dark green color indicating the viable nature of these cells. HepG2 cells treated with SIL significantly induced a shift from viable cells to

EB positive late apoptotic or dead cells. A concentration-dependent increase in the EB positive apoptotic cells was observed in the EBLE treatments (Figure 4A). Therefore, EBLE treatments showed percent- age increase of apoptotic cell population was markedly based on con- centration dependent (Figure 4B).



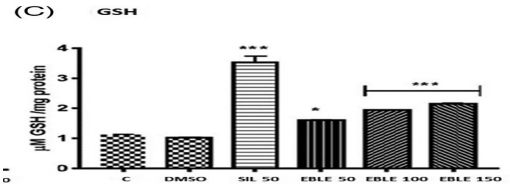


Figure 3: Ethanolic banaba leaves extract (EBLE)–induced changes in oxidative stress markers. (A) Lipid peroxidation (LPO), (B) superoxide dismutase (SOD), (C) reduced glutathione (GSH). C, control; SIL, Silymarin 50 μg/mL. Data presented as mean ± SEM (n = 3). ***P < .001 vs control

EBLE treatments induced changes in the ΔΨm in HepG2 cells

EBLE treatments induced changes in the ΔΨm of HepG2 cells are presented in Figure 5A. Control and DMSO-treated cells did not undergo shift from JC-1 dimer to JC-1 monomer and hence, appeared as red colored cells. SIL and high EBLE concentrations (100 and 150 μg/mL) treated cells showed red to green color change suggesting the shift from JC-1 dimer to JC-1 monomer. However, low- concentration of EBLE treatments produced lesser effect than the higher concentrations. Quantification of ΔΨm was found to be decreased in SIL and EBLE treated cells as compared to control (Figure 5B).

EBLE treatments induced changes in apoptotic marker genes

Furthermore, to investigate the molecular mechanisms behind the cyto- toxic effect of EBLE, we investigated apoptosis related marker genes pertaining to mitochondrial pathway. EBLE (100 and 150 μg/mL) and SIL treatments in HepG2 cells caused significant ($P < .001$) diminution of Akt expression. However, EBLE in low concentration did not produce any significant change in Akt expression as compared to control. EBLE and SIL treatments induced significant ($P < .001$) increase in the expres- sion of Bax with concomitant decrease ($P < .001$) observed in bcl-2 expression. In low concentration (50 μg/mL), EBLE treatments did not alter Bax expression, whereas on the other hand, bcl-2 was significantly ($P < .01$) decreased. Furthermore, EBLE and SIL treatments significantly($P < .001$) increased the expressions of cytochrome c, Apaf-1, and caspases 9 and 3. However, in lower concentration (50 μg/mL), EBLE treatments caused marginal expression of Apaf-1 ($P < .05$) and caspase 3 ($P < .01$) (Figure 6).

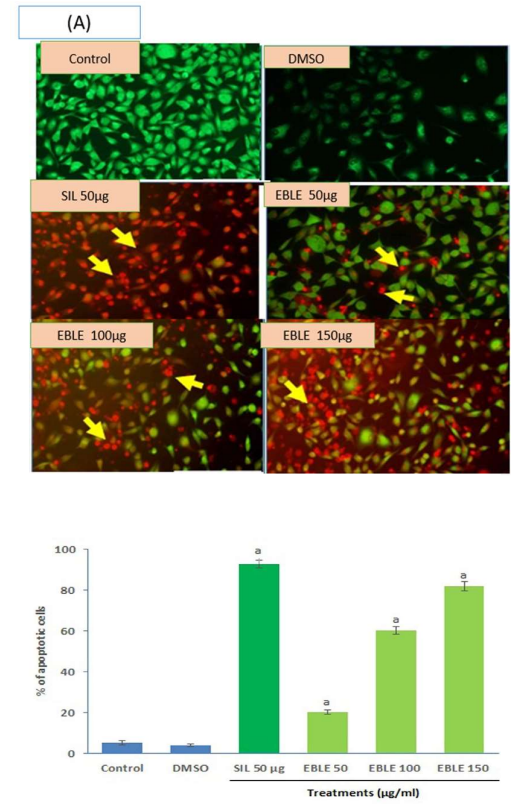
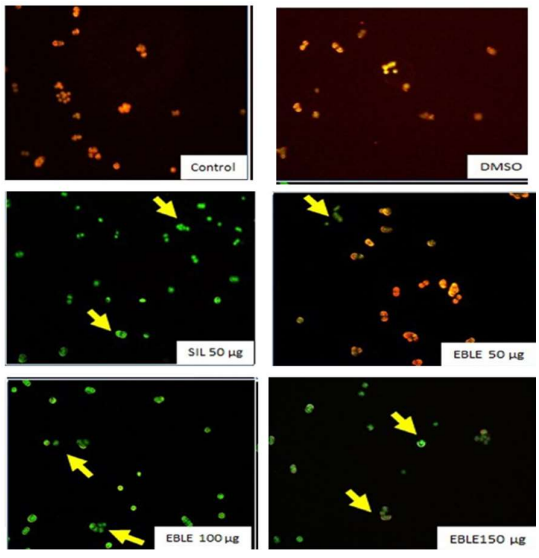


Figure 4. Ethanolic banaba leaves extract (EBLE)–induced morphology-related apoptosis. (A) Representative pictures from Dual staining (10×). Yellow arrow shows apoptotic cells. (B) Quantification of apoptotic cells. Data represented as mean ± SEM (n = 3). a P < .001 vs control



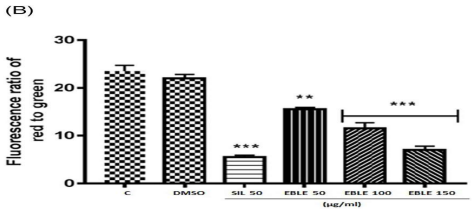


Figure 5: Ethanolic banaba leaves extract (EBLE)–induced changes in mitochondrial membrane potential in HepG2 cells. (A) Representative pictures from JC-1 staining. Yellow arrows show HepG2 cells with impaired mitochondrial membrane. (B) Quantification of MMP. Data represented as mean ± SEM (n = 3). $^{**}P < .01$, $^{***}P < .001$ vs control

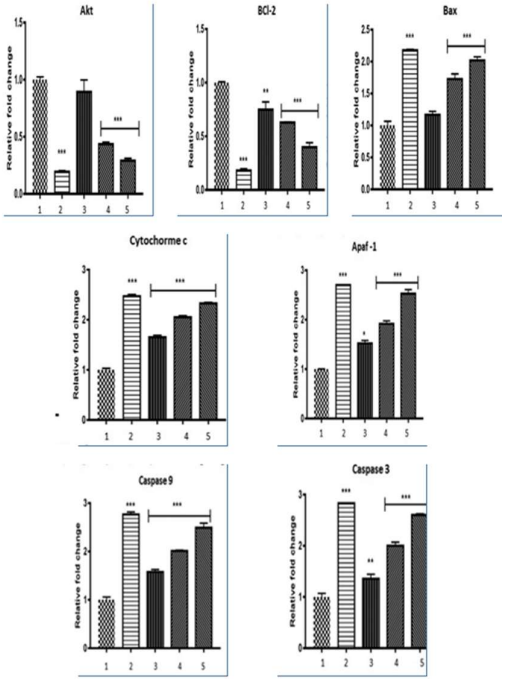


Figure 6: Ethanolic banaba leaves extract (EBLE)–induced changes in relative mRNA expressions of apoptotic marker genes. Values are expressed as mean ± SEM (n = 3). **P < .01 and ***P < .001 vs control. 1, control; 2, SIL 50 µg/mL; 3-5, EBLE 50, 100, 150 µg/mL treatments.

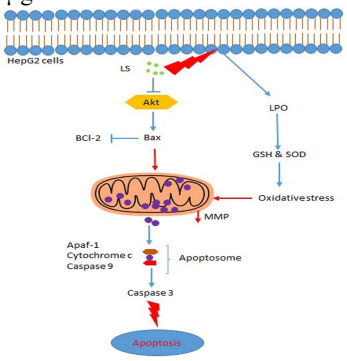


Figure 7: Probable mechanism of ethanolic banaba leaves extract (EBLE)–induced intrinsic (mitochondrial) pathway of apoptosis in HepG2 cells. LPO, lipid peroxidation; GSH, reduced glutathione; MMP, mitochondrial membrane potential.

DISCUSSION

Herbal medicines have emerged as an attractive treatment option for HCC management due to their immunomodulation and interactions with a variety of cancer cell signaling.[24] It has been reported that 70%-95% of the people in developing countries depend on traditional medicines.[25] Several plant-derived chemotherapeutic drugs such as paclitaxel (Taxol), camptothecin, podophyllotoxin analogues, vincris- tine, vinblastine, and many others are currently used in patients with different types of cancers.[25,26] These plant-derived anticancer drugs have the ability to induce cancer cell death via intrinsic or extrinsic apoptotic pathways and thereby offering anticancer effects.[27] The present study revealed the presence of active phytocompounds like berberine, and gallic acid in EBLE. These phyto-compounds are established independently as cytotoxic agents against a variety of can- cer cell lines including HepG2 cells.[14,28-30] Recently, studies have explored the cytotoxic potentials of ethanolic and methanolic banaba leaves extract against breast cancer cell lines.[31,32] Therefore, it is suggested that regardless of the cell line, EBLE induced cytotoxicity in HepG2 cells, and this could be due to the presence of above phyto- constituents.

Studies have shown that medicinal plants and their derived phytocompounds could induce oxidative stress-mediated cytotoxicity in several cancer cells.[33,34] Therefore, to find out the reason behind the EBLE-induced cytotoxicity, we investigated the oxidative stress markers. This study found that EBLE and SIL treatments induced oxidative stress as evidenced by an elevation of LPO and the corresponding increase in the primary first-line antioxidant defense such as SOD and GSH. Increased ROS production by EBLE treatments were directly correlated with the elevation of LPO.[35] Studies have shown that induction of LPO and activation of intracellular antioxi- dant enzymes by plant-derived compounds leading to oxidative stress is one of the strategies to induce cytotoxicity in cancer cells.[33] Oxida- tive stress and along with elevation of GSH could probably be due to increased NADPH availability, a cofactor of GSH and component of GSH synthesis as suggested by Arumugam et.[36] Furthermore, it is suggested that when cells are under oxidative environment, the anti- oxidant machinery is induced to nullify the intracellular oxidative stress condition. Thus, the enhanced levels of LPO, GSH, and SOD observed upon the exposure of EBLE and SIL in HepG2 cells indicate the

oxidative stress.

AO/EB staining is usually performed to assess the appoptosis- associated nuclei fragmentation, nuclear chromatin condensation and degradation, DNA condensation, and so on.[4,37] When the cells are underwent apoptotic changes, the nuclear chromatin changed from green to ethidium bromide positive red, indicating that EB moved to the nuclei via damaged cell membrane which lost its integrity and sta- ined nuclei red.[37,38] In light of the above reports, the present study shows increased red-colored nuclei in the EBLE and SIL treatments indicating the cells with loss of membrane integrity and morphological changes related to apoptosis.

In general, mitochondria are known to play a central role in the apoptotic process.[39] Reduction of ΔΨm is correlated with mitochon- drial dysfunction that frequently occurs in early apoptosis. Cancer cell mitochondria are highly susceptible to medicinal plants and their derived compounds which cause ΔΨm loss.[40,41] The fluorescent dye, JC-1, aggregates into healthy mitochondria and emits red fluores- cence. Upon ΔΨm loss in apoptotic cells, there is a shift from red to green fluorescence.[42] In accordance with this, we found that EBLE and SIL treatments disrupted the ΔΨm as observed by the increased intensity of green fluorescence.

Apoptosis is a programmed cell death, and many plant-derived drugs induce anticancer effects via mitochondrial apoptosis in cancer cells.[43,26] Therefore, in this study, we investigated molecular markers pertaining to intrinsic mitochondrial-mediated apoptosis pathway. Akt is responsible for cell proliferation, apoptosis-resistant, and cell cycle progression of cancer cells.[44] Activation of Akt expression has been widely reported during the cancer progression.[45] In this study, EBLE treatments inhibited the proliferation of HepG2 cells, and this could be due to downregulation of Akt gene expression. Bax and Bcl-2 are pro and anti-apoptotic genes, respectively, that regulate the intrinsic apoptotic pathway.[46] It has been reported that Bax and Bcl-2 expres- sions were altered during the early stages of apoptosis.[37,47] Further- more, dissipation of ΔΨm is responsible for the release of cytochrome c into the cytosol, where it combines with caspase 9 and Apaf1 forms apoptosome.[48] This apoptosome complex subsequently resulted in the activation of executioner of apoptosis, that is, caspase 3 which triggers apoptosis.[1] Therefore, it could be stated that EBLE treatment in HepG2 cells altered the homeostasis of Bax/Bcl-2 ratio, caused ΔΨm loss, released cytochrome c, and formed apoptosome in the cytosol that triggers apoptosis via caspase 3 activation (Figure 7).

In conclusion, EBLE treatment caused significant cytotoxicity in HepG2 cells. This treatment also caused an accumulation of lipid peroxi- dation products and a concomitant increase in the intracellular enzymic and non-enzymic antioxidants. Enhanced LPO caused apoptosis-related morphological changes in HepG2 cells. Furthermore, EBLE treatments reduced Akt expression and modulated the Bax/Bcl-2 homeostasis and altered the ΔΨm which in turn caused a dislocation of cytochrome c to the cytosol and activation of caspases that triggers apoptosis.

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