Journal of Applied Biology & Biotechnology Vol. 12(3), pp. 176-184, May-Jun, 2024
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2024.168741

Triphala extract Reduces Anti-apoptotic Protein (Mcl-1) Expression through the Activation of Caspase 3 in Hepatocellular Carcinoma Cells

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ARTICLE INFO

Article history:
Received on: December 08, 2023
Accepted on: February 23, 2024
Available online: April 20, 2024

Key words:
Triphala,
Hepatocellular carcinoma,
HepG2,
Hep3B,
3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay,
Apoptosis.

ABSTRACT

Triphala is an equal mixture of the three myrobalans *Terminalia chebula* (Haritaki), *Terminalia bellerica* (Bibhitaki), and *Phyllanthus emblica* (Amalaki), according to the Ayurvedic Formulary of India. Triphala is an herbal supplement that is high in antioxidants and has a number of advantageous qualities. Numerous investigations conducted in recent years have shown that triphala has anti-mutagenic and radioprotective properties. Therefore, the aim of the present study was to examine if an ethanolic extract of triphala had any inhibitory effects on hepatocellular carcinoma cells (HCCs). Gas chromatography-mass spectrometry analysis was used to identify and quantify various photochemicals in triphala extract (TE). Further, in two human liver cancer cells (HepG2 and Hep3B), TE was examined for its cytotoxic potential by [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Apoptosis was promoted by TE, which also suppressed the production of anti-apoptotic proteins and triggered poly-ADP ribose polymerase cleavage. It's significant that TE has a cytotoxic effect against liver cancer cells. The current study demonstrated that the ethanolic extract of triphala has anticancer properties against HCC. As a result, triphala may offer a potential alternative to conventional therapy for cancer patients.

1. INTRODUCTION

Triphala is a well-known triherbal ayurvedic medicine. *Phyllanthus emblica* (Amalaki), *Terminalia chebula* (Haritaki), and *Terminalia bellirica* (Bibhitaki) are the three herb fruits that make up this blend. Secondary metabolites in *Phyllanthus emblica* include ascorbic acid, polyphenols, tannins, flavonoids, terpenoids, glycosides, and many other individual compounds such as chebulinic acid, gallic acid, corilagin, geranin, quercetin, and furosin [1,2]. Flavonoids, phenolic acids, and tannins were among the bioactive chemical ingredients of Haritaki identified by Nigam *et al*. 2020 [3]. Bibhitaki also contains tannins, gallic acid, flavonoids, glucosides, terpenoids, terpene acids, saponins, glycosides, lignans, chebulinic acid, ellagic acid, vitamin C, and bellaric acid as key bioactive compounds. Kumar and Khurana (2018) [4] discovered that Bibhitaki contains antioxidant and anticancer properties, in addition to other therapeutic potential triphala has been widely utilized in the traditional Ayurvedic medicine of India to treat a variety of hepatic, gastrointestinal, and cardiovascular conditions [5]. Triphala has numerous medicinal effects, including antioxidant, anti-inflammatory, anticancer, immunomodulatory, and antimutagenic properties [1]. Notably, the anti-cancer potential of this traditional treatment has also been reported for different types of cancer.

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Previous research findings has demonstrated the chemoprotective, antineoplastic, and radioprotective properties of triphala [6]. Acute and chronic toxicity testing on healthy volunteers and animal models was used in various papers to validate the safety of Triphala [7-9].

Reactive oxygen species (ROS) promote oxidative stress with endogenous molecules such as nucleic acids, lipids, lipoproteins, and proteins, which causes cellular damage or tissue injury [10]. Most notably, ROS-induced cellular injury is implicated in cancer, neurodegenerative illnesses, inflammatory illnesses, and cardiovascular diseases [10,11]. Cancer and liver damage have been linked to the use of synthetic antioxidants in the food sector [11]. As a result, discovering safe and novel antioxidants from natural origin, such as plants, is critical. Many plants contain antioxidants such as tannins, flavonoids, phenolic acids, and polyphenolic chemicals. These compounds can reduce excess ROS to prevent ROS-induced illnesses in our body due to their metal chelating properties, redox effects, hydrogen-donating power, and singlet oxygen quenching capacity [10,11].

In prior work, Triphala and its components showed a strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging effect [12]. Several researchers investigated the ROS scavenging property of triphala and discovered that it eliminated radiation-mediated (X- and ɣ-) ROS generation in HeLa cells by scavenging superoxide free radicals and DPPH [13].

Triphala extract (TE) has been reported to have anticancer curative properties against a number of cancers both *in vitro* and *in vivo*,

including transplantable mouse thymic lymphoma barcl-95 [14], MDA-MB-231[15], Michigan Cancer Foundation (MCF-7) cell lines (breast cancer) [14,16], cervical adenocarcinoma [15], pancreatic adenocarcinomas (PANC-1) [15,17], colorectal carcinoma cell lines [18], human gastric cancer cells [19], and hepatocellular carcinoma (HCC) [20]. By activating the mitochondrial apoptotic signaling system, the *T. chebula*, *T. bellirica*, and *P. emblica* extracts demonstrated anticancer efficacy on cholangiocarcinoma cell lines [21]. In spite of this, numerous research using cancer cell lines and animal models point to its anti-cancer potential. In the current work, we were able to pinpoint the major bioactive components of triphala and investigate its potential cytotoxicity against HCCs.

2. MATERIALS AND METHODS

2.1. Preparation of TE

Triphala powder was used from a commercially available bottle. The alcoholic extraction of triphala powder was carried out by vortexing, mixing, and incubation (at 4°C) overnight in a shaking incubator. Extract was collected and centrifuged (4000 rpm) for 10 min after incubation. A 0.45 µm Poly tetra fluro ethylene syringe filter was used to filter the supernatant before it was collected, concentrated, and used in a nitrogen evaporator.

2.2. Cell Lines and Their Culture

Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (Sigma), penicillin (100 µg/mL), and streptomycin (100 µg/mL) was used to cultivate human liver cancer cells (HepG2 and Hep3B). A humidified environment with 5% CO_2 was used to keep cells at a constant 37°C temperature. For all *in vitro* experimental techniques, cell cultures with a confluence of around 80% were employed.

The equivalent ratio of individual components of TE was used for the analysis of cytotoxicity by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) [3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] test in HepG2 and Hep3B (human liver cancer) cell lines.

2.3. Analysis of Cytotoxicity of TE by MTT Assay

Following a procedure previously reported by Pandey *et al*. 2017 [22], the impact of TE on cell viability was determined using an MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test in HepG2 and Hep3B (human liver cancer) cell lines. In a nutshell, 5000 human HCCs were grown in triplicate in 96-well plates and subjected to a range of treatments for 72 h at 37°C [22]. Freshly made 20 µL of a MTT solution (5 mg/mL) was added after the initial 72 h of incubation. Formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide to each well of the 96-well plates after the supernatant had been centrifuged and discarded. The optical density of the resultant solution was calculated using the delta value (570–630 nm) on a microplate reader (96-well multi-mode) (BioTek Technologies, Vermont, USA). Prism software was used for the calculation of inhibition concentration (IC50) values by non-linear regression analysis.

2.4. Analysis of Phytoconstituents of TEs by Gas Chromatography-Mass Spectrometry (GC-MS)

For the GC-MS analysis, TE was dissolved in alcohol and injected in an GC-MS QP2010 model (Shimadzu®), Column (GC), SH-I-5Sil MS Capillary (30 m × 0.25 mm × 0.25 um), injection mode: splitless. For the analysis of the GC-MS set, the operating conditions were: temperature of the oven at 45°C for 2 min, 140°C for 5 min, and finally maintained at 280°C and held isothermally for 10 min.

The carrier gas was helium at a rate of 1 mL/min, and the sample injection volume was 2 L. The sample's constituent parts were ionized at a 70 eV energy level. The GC ran from 9.10 min to 52.0 min. The structures of the compounds were then compared to those in the NIST database using a search of the NIST14.L library (2020). The identification of the compounds was then done using the retention times and mass spectra of the compounds that were already known to exist in the NIST library (C: DatabaseNIST14.L) [23].

2.5. Annexin-V Assay

We employed a Muse® Cell Analyzer (Luminex Corporation, TX, USA) to analyze apoptosis. Prior to being put through a Luminex annexin V (live and dead) assay (Luminex Corporation, TX, USA) solution, HCCs were treated with TE for 24 h. The manufacturer's procedure for the annexin V (live and dead) assay was largely followed. In a nutshell, 1×10^5 cells were used per well overnight, followed by treatment and then incubation for 24 h. Cells were then scraped, combined with Annexin V dye (Luminex Corporation), and let to develop for 20 min. Data analysis was done using the Muse Cell Analyzer. Live cells can be observed in the lower left quadrant, which is annexin V negative, and early apoptotic cells may be seen in the lower right quadrant, which is annexin V positive and 7-amino-actinomycin negative (7-AAD). Late apoptotic and dead cells are present in the top right quadrant (annexin V+ and 7-AAD+). In the upper left quadrant, dead cells are shown by annexin V and 7-AAD+.

2.6. Western Blotting

In a 6-well plate with growth medium, 1×10^6 human liver cancer cells were sown overnight. The cells were treated with TE (0–50 mg/mL), vehicle control, and incubated for 24 h in serum-free medium. Using radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor cocktails, cells were washed and lysed after incubation [24]. After 1 h of incubation on ice, cell extracts were vortexed, and the cell lysate was centrifuged at 15,000 rpm for 10 min. The supernatant was kept at −80°C until future use. The amount of protein was calculated using a bicinchoninic acid protein assay kit (Pierce Scientific, IL, USA). Heat-denatured protein samples were separated using a 4–12% NuPAGE gel (Thermo Scientific, IL, USA). Proteins were transferred to PVDF membranes for 90 min at 30 volts. Membranes were blocked with 5% non-fat dry milk for 1 h before being incubated with primary antibodies overnight at 4°C. After that, membranes were exposed to goat polyclonal anti-actin antibody (1:5000, Santa Cruz Biotechnology) or rabbit monoclonal antibodies for poly ADP ribose polymerase (PARP), caspase 3, BCl-2, Mcl-1, and Bcl-xL (1:1000, Cell Signaling Technology) for an overnight incubation in a cold room. Membranes were then probed for 2 h at room temperature with secondary antibodies (HPR-conjugated), either goat anti-mouse or goat anti-rabbit, following a wash in TBS containing 0.1% Tween 20. Images were taken using a Bio-Rad imager after the membranes had been washed with TBS and developed.

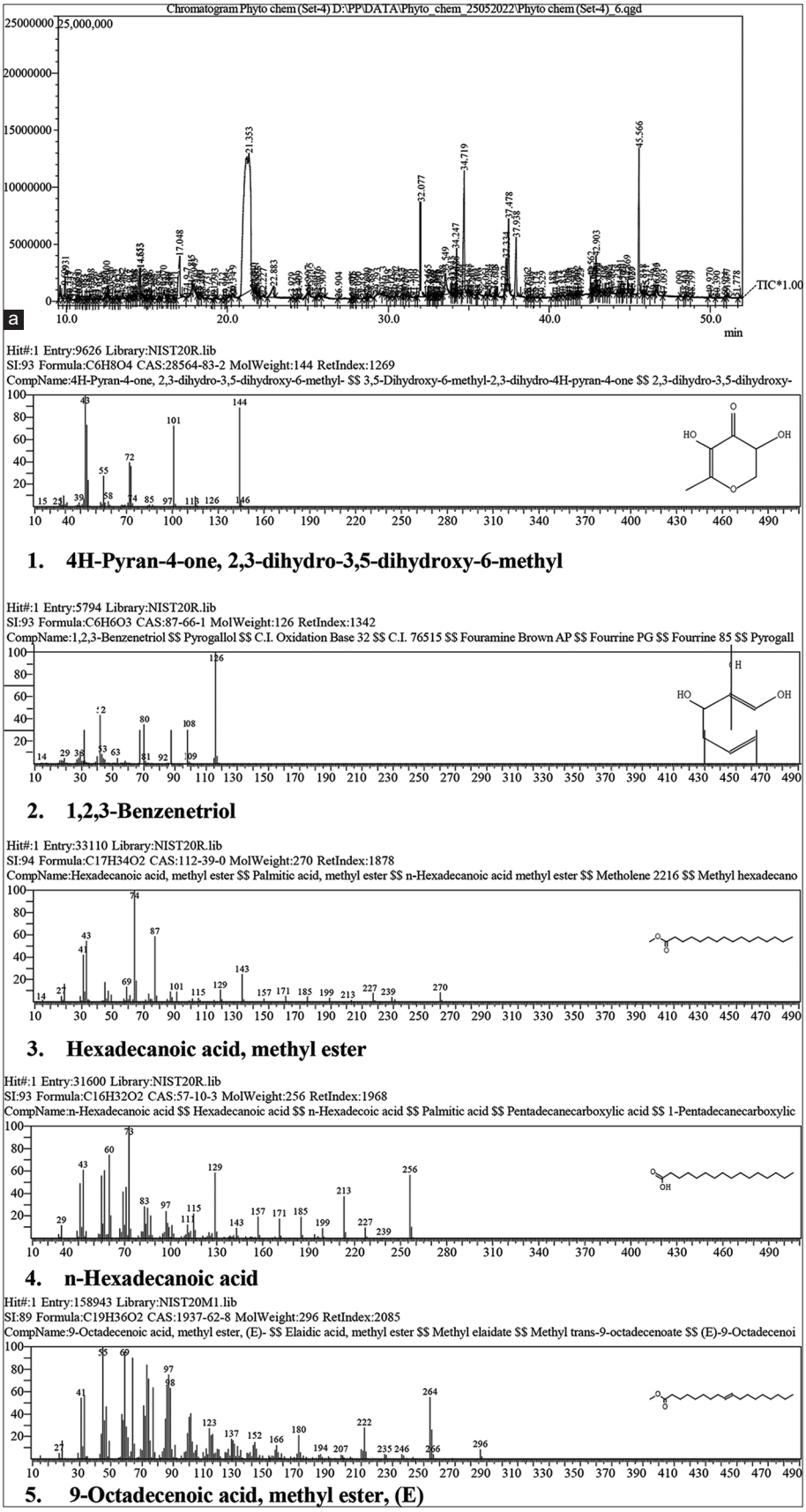
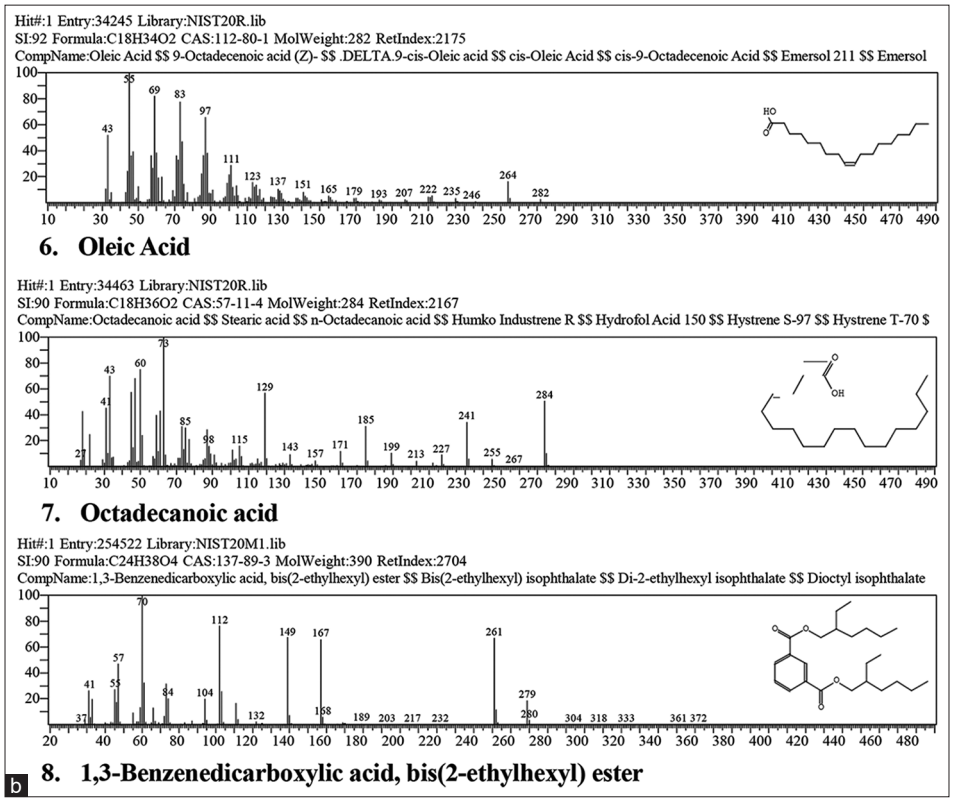


Figure 1: (a) Gas chromatography-mass spectrometry (GCMS) chromatogram of ethanolic extract of triphala. (b) Structure of phyotochemical compounds present in the ethanolic extract of triphala using GCMS analysis.



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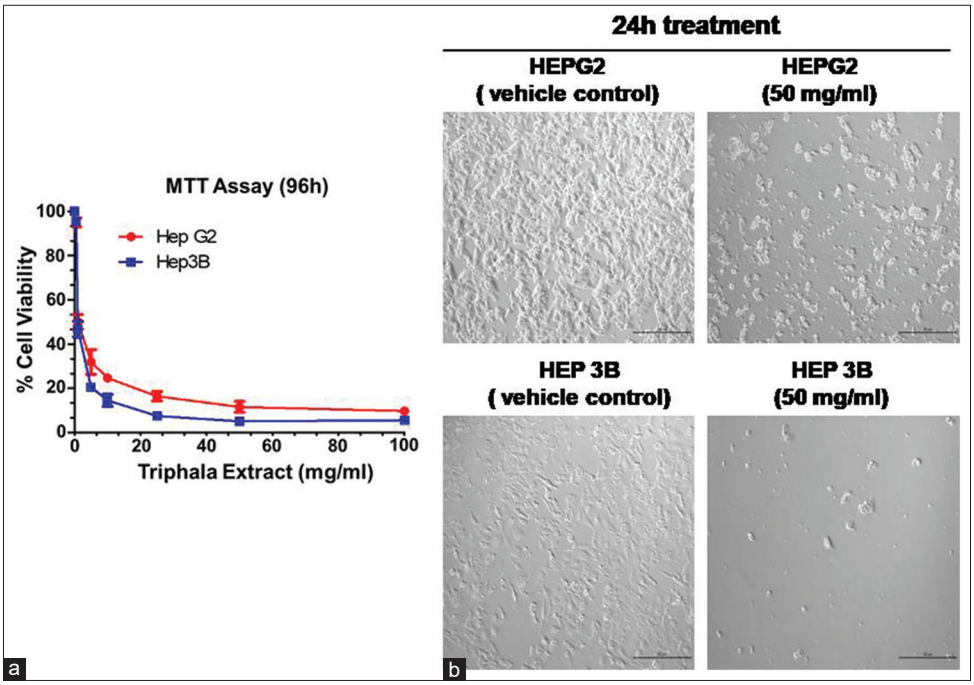

Figure 2: Triphala extract is cytotoxic against hepatic cancer cells. (a) HepG2 and Hep3B cells were treated with increasing amount of TE (1–100 mg/mL) for 96 h. Cell viabilities were determined by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay (b) HepG2 and Hep3B cells were treated with 50 mg/mL for 24 h and photographs were taken.

2.7. Statistical Analysis

Each experiment was performed in triplicates (n=3) for the MTT colorimetric assay and in duplicates (n=2) for the experiments that measured the cytotoxic activity. All statistical analysis, including calculating the mean and standard deviation and creating graphs, was done using Graphpad Prism.

3. RESULTS

3.1. The Ethanol extract of Triphala Contains a Variety of Phytochemicals

We looked into the phytochemicals in the ethanol extract of triphala. The GC-MS analysis was used to quantify these phytochemicals. As indicated in Figure 1a, we found 174 compounds, out of which 31 important compounds are listed in Table 1 and Figure 1b. Numerous compounds were discovered, including 1,2,3-benzenetriol (29.11), n-Hexadecanoic acid, 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, (Z)-3-Phenyl-2-propenoic acid, benzoic acid, 3,4,5-trihydroxy-methyl ester, and 9-Octadecenoic acid, (E)-methyl ester. Other important substances were phenol, catechol, 5-hydroxymethylfurfural, tetradecanoic acid, benzoic acid, 3,4,5-trihydroxy-, methyl ester, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, dimethyl (R)-(+)-malate, furyl hydroxymethyl ketone, octadecanoic acid, oleic acid, l-(+)-ascorbic acid, 2,6-dihexadecanoate, and vitamin E. A sizable number of substances from the chemical families of alcohol, fatty acid ester, aminoglycoside, fatty acid triterpene, etc. were identified and quantified in TE.

3.2. TE is Cytotoxic against HCC

By completing the MTT assay as specified in the above methodology section, the cytotoxic capability of TE was assessed against two HCCs (HepG2 and Hep3B). HepG2 and Hep3B hepatic cancer cell lines were given TE treatments at various concentrations (0–100 mg/mL) over the course of 96 h. Figure 2a demonstrates how both cell lines were successfully suppressed by TE, which had an IC_{50} of 2–5.0 mg/mL at 96 h. When compared to HepG2 cells, Hep3B cells were more susceptible to TE. Additionally, we looked into whether exposure to a higher concentration of TE for a brief period of time is cytotoxic. As shown in Figure 2b, lower panel, the higher dose of TE (50 mg/mL), which we found to be most effective within 24 h of therapy, is cytotoxic.

3.3. TE induces the apoptosis in hepatic cancer cells

HepG2 and Hep3B cells were exposed to progressively higher TE concentrations for 24 h [Figure 3] to determine if TE-induced cytotoxicity causes apoptotic cell death. Using the Muse Annexin V

Table 1: Phytochemical compounds identified in the ethanolic extract of triphala using GC-MS analysis.

S. No.	Compounds	Retention time (min)	Area	Height	A/H
1	Phenol	9.609	4681629	1172817	3.99
2	2H-Pyran-2,6 (3H)-dione	9.931	7301523	2002267	3.65
3	2,5-Furandione, dihydro-3-methylene	10.730	1216661	407389	2.99
4	3-Hydroxy-2-methylbutyricacid, ethyl ester	11.508	1876481	497344	3.77
5	Furyl hydroxymethyl ketone	12.600	3104159	1059579	2.93
6	3,4-Dimethyl-1,2-cyclopentadione	13.132	1559924	479092	3.26
7	Dimethyl (R)-(+)-malate	13.917	1303250	392341	3.32
8	Ethanamine, N-ethyl-N-nitroso	14.208	1076354	295674	3.64
9	Glutaric acid, cyclohexylmethyl 4-chloro-2-methoxyphenyl ester	14.553	11432045	2471203	4.63
10	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	14.615	9345993	2511063	3.72
11	Heptanoic acid, 6-oxo-, ethyl ester	15.133	336034	129948	2.59
12	Catechol	16.070	5101016	755442	6.75
13	5-Hydroxymethylfurfural	17.048	25900885	3642783	7.11
14	1,2,3-Benzenetriol	21.353	351999451	12090030	29.11
15	(Z)-3-Phenyl-2-propenoic acid	22.883	10647232	968283	11.00
16	Benzoic acid, 3-hydroxy-	25.115	1288233	308396	4.18
17	2,4-Di-tert-butylphenol	25.676	2044956	474524	4.31
18	1-Nonadecene	28.690	1122875	477189	2.35
19	Tetradecanoic acid	32.077	52052228	8327119	6.25
20	Benzoic acid, 3,4,5-trihydroxy-, methyl ester	33.549	15952466	1859159	8.58
21	Hexadecanoic acid, methyl ester	34.186	1481380	937051	1.58
22	n-Hexadecanoic acid	34.719	49499864	10690697	4.63
23	l-(+)-Ascorbic acid 2,6-dihexadecanoate	36.051	1345697	380720	3.53
24	Heptanoic acid, anhydride	36.284	1817119	638410	2.85
25	9-Octadecenoic acid, (E)-methyl ester	36.728	2314714	813894	2.84
26	Oleic acid	37.478	23745488	6020209	3.94
27	Octadecanoic acid	37.938	22905163	5167807	4.43
28	1,3-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester	45.129	117039	68486	1.71
29	13-Docosenamide, (Z)-	45.566	41325267	12786878	3.23
30	Cholesta-4,6-dien-3-ol, (3.beta.)-	49.970	1220599	278273	4.39
31	Vitamin E	51.077	1816479	404877	4.49

GC-MS: Gas chromatography-mass spectrometry

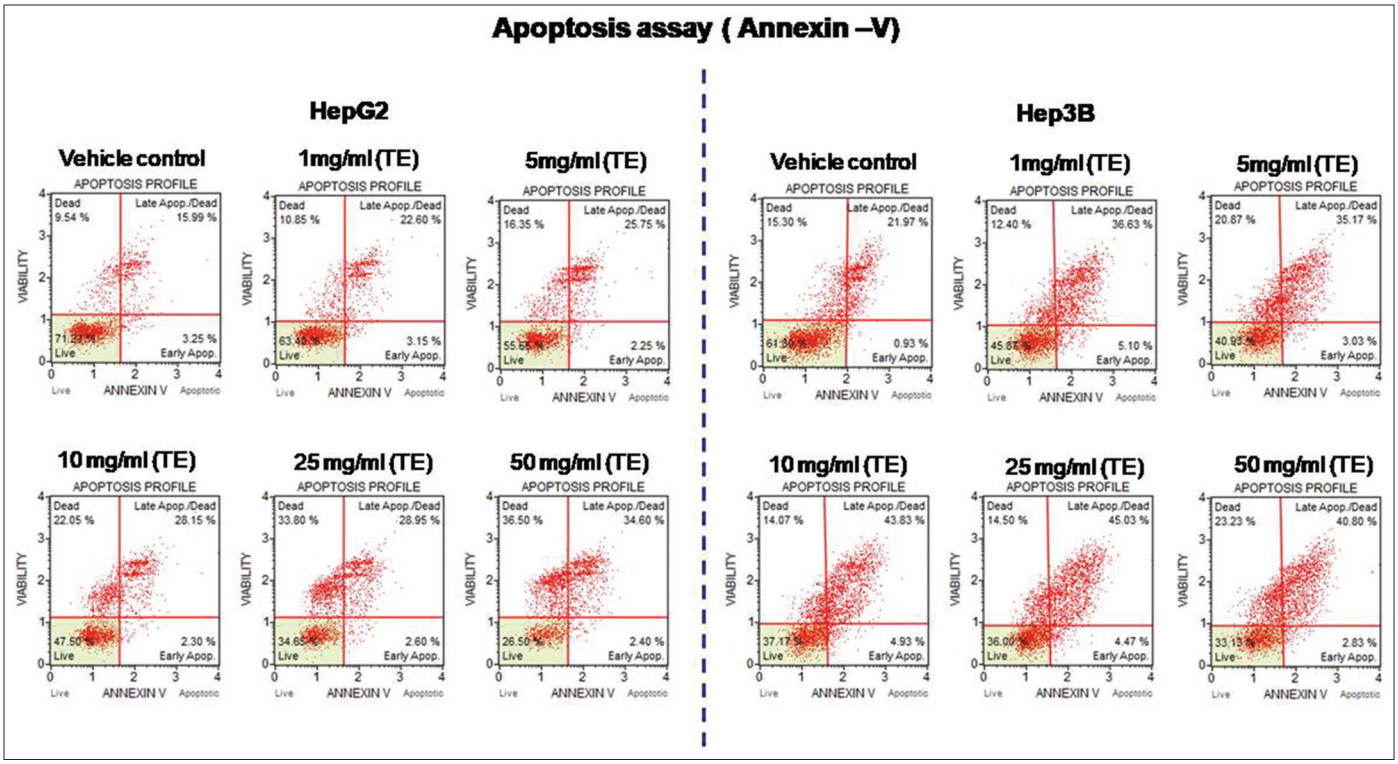

Figure 3: Triphala extract causes apoptosis in hepatic cancer cells. TE was added to HepG2 and Hep3B cells at various concentrations for 24 h, and the Muse apoptotic test was carried out as explained in the methodology section. Healthy cells are represented by the lower-left quadrant; early apoptotic cells are represented by the lower-right quadrant; late apoptotic cells are represented by the top-right quadrant; and necrotic cells are represented by the top-left quadrant.

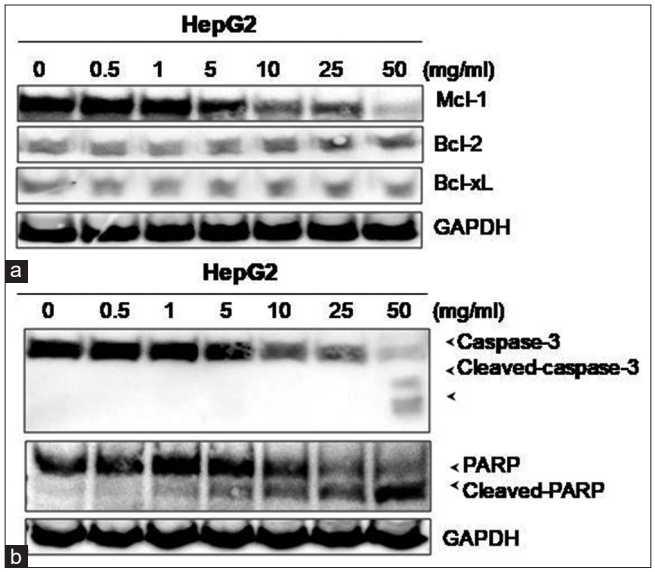

Figure 4: Triphala extract induces apoptosis by modulation of Mcl-1, caspase 3 and PARP. (a) TE had no effect on Bcl-2 and Bcl-xL, nonetheless inhibits Mcl-1. (b) TE induces PARP cleavage in a Caspase-3 dependent manner. HepG2 cells were exposed to TE for 24 h. Following incubation, cells were collected, total lysates made and fractions separated. The specified antibodies were used for the western blot analysis, and the same blots were examined again with either different antibodies or anti-GAPDH to assess loading, as explained in the methodology section.

test, apoptosis was found. Phosphatidyl serine (PS) is a substance that is found on the plasma membrane's outer surface and is one of the telltale signs of apoptosis. The Muse kit comprises the dye annexin V, which binds to PS on the plasma membrane's outer surface and recognizes apoptotic cells. The kit includes the staining agent 7-AAD, which is used to identify cells whose plasma membrane integrity has been compromised. The population of cells that test negative for annexin V (−) and 7-AAD (−) are therefore normal cells, and they will appear in the lower left quadrant of the graph. The lower right quadrant of the graph shows the presence of a cell population that is positive for annexin V but negative for 7-AAD, which are thought to be early apoptotic cells. The upper right quadrant of the graph, which indicates cells that died via apoptosis, shows a cell population that is positive for annexin and 7-AAD. Necrotic cells (seen in the upper left quadrant) are defined as cell populations that are only positive for 7-AAD and negative for Annexin V. Figure 3 illustrates how, in comparison to vehicle control, the amount of TE enhances apoptosis. According to this information, TE causes HCCs to die in an apoptotic way, which may be seen by the presence of annexin V binding on the plasma membrane's outer surface.

3.4. TE Reduces Anti-Apoptotic Protein (Mcl-1) Expression and Induces PARP Cleavage through the Activation of Caspase 3

Investigations were conducted on how TE affected the expression of Bcl-2 proteins that prevent apoptosis. The fact that TE suppressed the production of Mcl-1 in a dose-dependent manner suggests that the apoptotic response to TE is mediated by the Mcl-1 protein [Figure 4a]. By examining the amounts of caspase activation (cleaved caspase 3) and degradation of PARP, which is a target of caspase 3 (the caspase family of enzymes), we were able to corroborate our findings. Caspase 3 activation and PARP degradation were enhanced as the TE dose was raised, as seen in Figure 4b. Our conclusion from the Muse Annexin V

assay is supported by these findings. So, according to our findings, TE has the ability to fight cancer.

4. DISCUSSION

Since ancient times, the oldest system of plant-based therapy in India—Ayurveda—has been used to prevent or inhibit a variety of cancers. Additionally, scientists are becoming more interested in studying complementary and alternative therapies for the treatment of cancer. According to "Charaka" and "Sushruta Samhitas," in the Ayurvedic notion, cancer is referred to as an inflammatory or non-inflammatory swelling and is mentioned as either "Granthi" (a little neoplasm) or "Arbuda" (a significant neoplasm) [25,26]. The three systems (Tridoshas-Vata, Pitta, and Kapha) in malignant tumors lose their ability to coordinate with one another, which results in tissue destruction and a severe condition. Tridoshas lead to an overly severe metabolic crisis, which promotes proliferation [26]. A flawless therapy for the malignancy is expected from the complementary and alternative medicine systems, despite the fact that mainstream cancer treatment is investigated to be plagued by drug-induced hazardous complexities. It is crucial to research the potent and safe Ayurvedic medicine's anti-cancer properties.

In India, there is a lack of data on HCC due to the country's mostly urban cancer registries and the fact that cancer is not a reportable disease. Unpublished data from numerous tertiary care facilities in India shows an increase in the incidence of HCC. With an age-standardized cancer mortality rate of 6.8 (5.4–8.1)/100,000 cases, liver cancer was the cause of 14,000 cancer deaths in India in 2010 [27]. Previous research findings showed that triphala has been used to treat gastrointestinal disorders and hepatoprotection, but research on its potential as an anti-cancer drug, particularly against HCC, is still in its infancy. Thus, the main phytochemical compounds in the extract and the anti-cancer activities of the Ayurvedic medicine triphala were examined in our study.

GC-MS analysis of our research findings showed that the ethanolic extract of triphala led to the identification of 174 compounds belonging to the triterpene, fatty acid ester, aminoglycoside, fatty acid, alcohol, etc. Octadecanoic acid, 1,2,3, benzenetriol, n-Hexadecanoic acid, 9-Octadecenoic acid (E)-methyl ester, hexadecenoic acid, methyl ester, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy,-6 methyl, have been identified for their antioxidant properties [28-32].

1,3-benzenedicarboxylic acid, a bis(2-ethylhexyl) ester, has been reported to employ antimutagenic properties against various cancer cell lines, including A549, HCT-116, MCF, MIAPACA, and PC3 [31,33]. Other constituents of triphala (chebulagic acid and chebulinic acid) also have antitumor activity against the human osteosarcoma cell line [34]. It is remarkable to mention that 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, 1,2,3-benzenetriol, and oleic acid have anticancer activity. Therefore, triphala may have anti-cancer effects, and our present research findings also confirm the previous studies.

It is also well documented from earlier studies that every extract from plants also contains a number of health advantages, such as *in vitro* and *in vivo* anticancer activities. In particular, *P. emblica*, one of the plant extracts included in the triphala formulation, had significant levels of phenolic, flavonoid, and tannin content, had an inhibitory impact on inflammation, and showed potent anticancer activity against cholangiocarcinoma cells. While it was discovered that the *T. bellirica* extract prevented cell cycle arrest in the S phase. Additionally, by triggering death through the mitochondrial apoptotic signaling system, these plant extracts reduced the development of cholangiocarcinoma cells [21]. Oral carcinoma of the squamous cells was found to be resistant to the antiproliferative effects of *T. bellirica.* Interestingly, ellagic, gallic, chebulinic, and chebulagic acids were identified as the primary constituents of triphala [35]. *T. bellirica* extract contains gallic acid, a bioactive substance that is responsible for its free radical scavenging and selective antiproliferative activity [36]. Patra *et al*. 2020 [37] reported that the ROS played a crucial role in controlling apoptosis and made it easier for mitochondrial apoptosis to damage DNA. According to Prasad and Srivastava 2020 [13], triphala has been shown to have an anticancer effect via altering a number of cell signaling pathways, including the mammalian targets of Rapamycin, c-Myc, Extracellular signal-regulated kinase (ERK), Mitogen-activated protein kinase (MAPK), NF-B, Protein Kinase B (AKT), p53, VEGFR, NF-B, tubulin, and cyclin D1 proteins that prevent and promote apoptosis. Triphala has antiproliferative and antimetastatic effects on human gastric cancer cells due to its ability to prevent EGFR, AKT, and ERK phosphorylation [19]. Chebulinic acid, a key component of triphala, has powerful pro-apoptotic, anti-migratory, and anti-proliferative effects on colorectal cancer cell lines via the phosphoinositide-3-kinase/AKT and MAPK/ERK pathways [18]. Triphala also has anticancer properties against breast cancer (MCF-7 cells). It was also found to have an anti-proliferative impact on PANC-1, MDA-MB-231 (triple-negative breast cancer), and cervical adenocarcinoma (HeLa) cells [15].

Using the MTT assay, we examined the triphala ethanol extract's capacity for cytotoxicity against HCCs. A colorimetric test called MTT is used to gauge cellular metabolism. The MTT colorimetric method is used to investigate how cytotoxic medicines affect cancer cell growth and proliferation and to calculate the IC_{50} of these substances. We discovered that TE was efficient on HCC and drastically decreased its survival rate, which was further verified by morphological examination. Accordingly, a number of studies have documented the cytotoxic effects of triphala, demonstrating a considerable reduction in cell proliferation [13,17]. On colon cancer cells (HCT116) and human carcinoma stem cells, Prasad and Srivastava 2020 [13] reported that triphala methanol extract has a dose-dependent antiproliferative effect.

By concentrating on anti-apoptotic proteins, we were able to show that TE causes apoptosis. It's significant that the TE was able to cause the caspase-dependent cleavage of PARP, which is a hallmark of cell death. Others have shown that TE causes colon cancer cells to undergo apoptosis by activating the mitochondrial apoptotic signaling system [38]. Additional research on a range of malignancies, including gastric, ovarian, breast, prostate, and pancreatic cancer, points to the efficacy of this Ayurvedic treatment. Overall, our research indicated that triphala has considerable cytotoxicity against HCC cells; therefore, more formulations of this extract must be standardized and tested *in vivo* models in order to finally improve their efficacy.

5. CONCLUSION

In our investigation, triphala dramatically reduced the proliferation of HepG2 and Hep3B cells, which is significantly correlated with previous findings. Our results confirm the anticancer properties of TE. Moreover, it is safe to use as an oral formulation. As a result, triphala may offer a potential alternative to conventional therapy for cancer patients.

6. ACKNOWLEDGMENT

This research was funded (grant no. 80/2021/1543/70-4-2021-4(28)/2021) by Higher Education, Uttar Pradesh State Government, India.

7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Mishra Y, Yadav RR, Verma K, Yadav N. Triphala extract Reduces Anti-apoptotic Protein (Mcl-1) Expression through the Activation of Caspase 3 in Hepatocellular Carcinoma Cells. J App Biol Biotech. 2024;12(3):176-184. DOI: 10.7324/JABB.2024.168741