Saudi Journal of Biological Sciences 29 (2022) 103287

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

GC-MS-employed phytochemical characterization, synergistic antioxidant, and cytotoxic potential of Triphala methanol extract at non-equivalent ratios of its constituents

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ARTICLE INFO

Article history:
Received 11 December 2021
Revised 8 March 2022
Accepted 17 April 2022
Available online 27 April 2022

Keywords:
Triphala
Nonequivalent ratio
Antioxidant
Cytotoxicity
Phytochemical characterization
GC-MS

ABSTRACT

Triphala is a famous triherbal drug, comprising three herb fruits, including *Terminalia chebula* (Haritaki), *Terminalia bellirica* (Bibhitaki), and *Phyllanthus emblica* (Amalaki). It is enriched with vitamin C, polyphenols, flavonoids, sterols, saponins, etc., and is well-documented for its potent antioxidant, anticancer, chemoprotective, antimicrobial, and anti-inflammatory effects. This research was conducted to evaluate the synergistic antioxidative and cytotoxic potential of mixtures of the individual constituents of Triphala at their nonequivalent ratios along with the chemical characterization of individual constituents of Triphala to identify and quantify individual compounds. The antioxidative potential was measured using total antioxidant capacity (TAC), DPPH free radical scavenging assay, and total phenolic content (TPC) tests. The cytotoxic potential was assessed on brain cancer cells (N4X4) using MTT assay, and phytochemical characterization was performed by GS-MS analysis. Nonequivalent ratios of Triphala constituents exhibited significantly higher synergistic antioxidant and cytotoxic potential than the equivalent ratios of them. Moreover, the nonequivalent ratio where the quantity of Amalaki was doubled than the other two constituents showed the highest synergistic antioxidant and cytotoxic effect. GC-MS analysis of individual constituents of Triphala identified and quantified the presence of a wide array of compounds, and fatty acid, fatty acid ester, triterpene, and aminoglycoside remained the predominant class of compounds. Thus, it can be inferred that the observed bioactivities can be attributed to the phytocompounds characterized and extracts at the nonequivalent ratio of Triphala constituents where Amalaki is doubled can be more effective in treating oxidative degenerative diseases and glioblastoma.

1. Introduction

Triphala, a popular triherbal drug, has traditionally been utilized in the Indian subcontinent to cure a wide array of disorders from ancient times. It comprises three herb fruits; *Phyllanthus emblica* (Amalaki), *Terminalia chebula* (Haritaki), and *Terminalia bellirica* (Bibhitaki). Amalaki contains biologically active secondary metabolites including vitamin C, polyphenols, flavonoids, glycosides, terpenoids, tannins, etc. and individual phytocompounds such as phyllemblin, gallic acid, geranin, furosin, corilagin, quercetin, chebulinic acid, and many more (Hasan et al., 2016; Saini et al., 2022). Amalaki has a huge therapeutic potential due to possessing these phytoconstituents that contribute to a wide range of bioactivities, including antioxidant, anticancer, anti-inflammatory, immunomodulatory, and antimutagenic (Saini et al., 2022). Different bioactive chemical constituents of Haritaki have been identified and reported, including flavonoids, phenolic acids, tannins, and other compounds. Bioactivities of Haritaki, such as antioxidant, cytotoxic, neuroprotective, and anti-inflammatory effects, among others, have been reported (Nigam et al., 2020). Bibhitaki contains flavonoids, glycosides, glucosides, tannins, vitamin C, terpenoids, saponins, lignans, gallic acid, chebulinic acid, ellagic acid, bellaric acid, terpene acids as its major bioactive chemical

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2022.103287
1319-562X/

constituents. Bibhitaki has enormous therapeutic potential, especially antioxidant and anticancer effects, among other bioactivities (Kumar & Khurana, 2018).

Reactive oxygen species (ROS) induces oxidative stress and lead to cellular damage or tissue injury by reacting with the endogenous molecules, including lipids, proteins, lipoproteins, and nucleic acids (Zhou et al., 2020). Most importantly, ROS-induced cellular damage is responsible for neurodegenerative diseases, cardiovascular diseases, cancer, and inflammatory diseases (Krishnaiah et al., 2011; Zhou et al., 2020). Synthetic antioxidants used in the food industry have produced cancer and liver damage (Krishnaiah et al., 2011). Therefore, it is important to discover new/novel and safe antioxidants from natural sources like plants. Plant extracts are potential antioxidants such as polyphenolic compounds, flavonoids, tannins, and phenolic acids. These compounds can neutralize excessive ROS in the body due to their possessing redox properties, metal chelating effects, singlet oxygen quenching power, and hydrogen donating ability and prevent ROS-induced diseases (Krishnaiah et al., 2011; Zhou et al., 2020).

A significant DPPH free radical scavenging was exhibited by Triphala and its constituents in a previous study conducted by Singh et al. (Singh et al., 2016). A number of researchers studied the ROS scavenging activity of Triphala and found that it obliterated X- and γ- radiation-mediated ROS production in HeLa cells and scavenged DPPH and superoxide free radicals (Prasad & Srivastava, 2020).

Brain tumors are the second most common cancer in children, and consist of around 40% glioma, about 15–25% of pediatric malignancies, and approximately 25% medulloblastomas (Hussain, 2013). Glioblastoma is one of the cancers with rapid progression and largely remains incurable despite advances in cancer treatment modalities (Hanif et al., 2017). Researchers found that herbal medicinal products are considered one of the best treatments for cancer (Kumar et al., 2012; Pham et al., 2018). Compared to synthetic drugs, these natural compounds are more readily available, cheaper, can be easily administered orally, and have negligible side effects (Seca & Pinto, 2018; Lichota & Gwozdzinski, 2018). A previous study showed that natural plant compounds could work as an anticancer agent and restore chemotherapy sensitivity. For example, during an in vivo study, the synergistic anticancer effect of doxorubicin towards resistant MCF-1/DOX cells was detected when a natural active alkaloid, tetrandrine, was used in combination with doxorubicin (Aung et al., 2017). Paclitaxel, a natural anticancer drug, was isolated from the *Texus bravefolia* plant. It has been most effectively used in the clinical treatment of numerous cancers, including lung, breast, and ovarian cancers (Pham et al., 2018). Moreover, identifying medicinal plants with potent cytotoxicity is the key to discovering and developing effective cancer therapy. Research is expanding day by day, searching for safe, effective, and more selective natural drugs for chemotherapy (Akter et al., 2014).

Several study findings reported significant inhibition of a vast number of cancer cells growth when the anticancer effect of Triphala was investigated against different cancer cell lines: Capan-2 pancreatic cells, gastric cancer cell, SKOV-3, HeLa, HEC-1B, colon cancer cells, PANC-1, breast cancer cells; MDA-MB-231 and MCF-7, HCT116, HCCSCs, PC-3, T47D, and DU-145, BxPC-3 and HPDE-6 and findings showed significant inhibition of all cancer cells growth (Prasad & Srivastava, 2020).

The available Triphala ayurvedic formulation used to treat various disorders contains Amalaki, Haritaki, and Bibhitaki in equal proportions. Formulation containing nonequivalent ratios of each constituent might provide a better therapeutic effect than the formulation containing three constituents at equivalent ratios due to varying in range and concentrations of phytocompounds present in each constituent. *In vitro* study is warranted to see whether Triphala extract comprising three constituents at their nonequivalent ratios produces a higher antioxidant effect before opting for formulation development. In addition to that, no study was performed on N4X4-brain cancer cells previously with Triphala extract. Thus, our present study selected methanol extract of Triphala for the synergistic antioxidant potential and cytotoxicity determination with N4X4 cell lines with nonequivalent ratios of its components. The positive findings of our current study might encourage formulation scientists to develop a new formulation of Triphala using nonequivalent ratios of each constituent.

2. Materials and methods

2.1. Fruit materials

Amalaki, Haritaki, and Bhibitaki fruit powders were collected from commercially available packets. Extracts of three fruits were obtained by cold maceration using methanol as a solvent. 600 gm of the coarse powder of Amalaki, Haritaki, and Bibhitaki was separately soaked in 1.2 L of methanol in three cleaned beakers and kept for seven days at ambient temperature with occasional stirring (22–25 °C) (Alam et al., 2013). After filtering, the filtrate was transferred to a round bottom flask and concentrated using a vacuum rotary evaporator (Heidolph, Germany), maintaining the temperature and speed at 30 °C and 100 rpm, respectively. The extract was preserved in the refrigerator at −20° C for further use.

2.2. Measurement of the antioxidative potential of Triphala

The antioxidative potential of Triphala was measured by employing three different tests such as DPPH free radical scavenging assay to identify its inhibitory potential on free radical scavenging, total phenolic content (TPC) test to determine the total quantity of phenols present, and total antioxidant capacity (TAC) test to assess its overall antioxidative potential. To perform TPC and TAC tests, a range of concentrations (200–1200 μg/mL) and for the DPPH assay, a range of concentrations (3.125–1200 of μg/mL) of Triphala was prepared by serial dilution.

2.2.1. DPPH free radical scavenging potential of Triphala

The antioxidant effect of Triphala extracts at different ratios of all three components, and its individual components were evaluated by its potential to scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radicals using the method described earlier (Lalhmingmawii & Jagetia, 2018). 0.5 mL of each extract solution of different concentrations was added and appropriately mixed with 1 mL of 0.1 mM methanol solution of DPPH. The solution mixture was kept in the dark for 30 min. The absorbance of the control (methanol) and sample solutions was measured at 523 nm using a UV–Vis spectrophotometer (U-2910, Hitachi High Technologies, USA), L-ascorbic acid served as the standard antioxidant. The calculation of the % inhibition of DPPH radical scavenging activity of Triphala was done utilizing the equation $[(A_o\text{-}A_1)/A_o]$ where A_o and A_1 are the absorbance of the control and sample/standard, respectively. IC_{50} values were calculated from the percentage inhibition curves.

2.2.2. Total phenolic content assay of Triphala

Estimation of total phenolic content (TPC) of Triphala extracts at different ratios of all three components and its individual components was performed by the modified Folin-Ciocalteu method described earlier (Chandra et al., 2014). 0.2 mL of the test sample was added with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent in a 1:1 ratio. 1 mL of 8% (w/v in water) saturated sodium carbonate solution was added to the previous solution after 5 min. The mixture was then kept in the dark for 30 min to

complete the reaction. Absorbance was measured at 765 nm utilizing a UV–Vis Spectrophotometer (U-2910, Hitachi High Technologies, USA). Gallic acid served as the standard, and TPC was calculated by extrapolating the calibration curve prepared by different concentrations of gallic acid solutions. The determination of the phenolic compounds was conducted in triplicates. In addition to this, the TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram of the dried extract (mg of GAE/g dried extract).

Gallic Acid Equivalents (GAE) were found using the following equation:

$$C = \frac{c \times V}{m}$$

Where, C = Total content of phenolic compounds, milligrams of Gallic acid per gram of dried plant extract, expressed as Gallic acid equivalents (QE).

Gallic Acid Equivalent (GAE) c = Concentration of Gallic acid obtained from calibration curve (mg/ mL).

V = Volume of sample solution (mL), m = Weight of the sample (g).

2.2.3. Total antioxidant capacity of Triphala

Estimation of total antioxidant capacity (TAC) of Triphala extracts at different ratios of all three components and its individual components was achieved using the method mentioned earlier (Pisoschi & Negulescu, 2012). 300 μL of each of the test samples of different concentrations were taken in test tubes, and 3 mL of reagent solution (0.6 M sulfuric acid, 0.028 M sodium phosphate, and 0.004 M ammonium molybdate) was added into the test tubes and incubated at 95 °C in a water bath for 90 min. Absorbance was measured at 765 nm using a UV–Vis Spectrophotometer (U-2910, Hitachi High Technologies, USA). Ascorbic acid was utilized as the standard, and TAC was calculated by extrapolating the calibration curve prepared by different concentrations of ascorbic acid solutions. The estimation of TAC was conducted in triplicates, and the TAC was expressed as milligrams of ascorbic acid equivalent (AAE) per gram of the dried extract (mg of AAE/g dried extract).

The total antioxidant capacity, A, for each of the fractions was expressed as:

$$C = \frac{c \times V}{m}$$

Where, C = Total antioxidant capacity, milligrams of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (AAE).

c = Concentration of ascorbic acid obtained from calibration curve (mg/mL).

V = Volume of sample (mL), m = Weight of the sample (g).

2.3. Cytotoxicity determination of Triphala by MTT assay

Cytotoxicity of Triphala extracts at the equivalent ratio (A:B:H = 1:1:1), and nonequivalent ratio (A:B:H = 2:1:1) of its individual components was determined against N4X4 (Human glioma NP2 derived) cell line utilizing the MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

2.3.1. Cell culture

At a temperature of 37 °C in a humidified incubator containing 95% air and 5% CO_2, the human glioma NP2- derived cell line, N4X4 was cultured and kept in advanced RPMI 1640 medium and DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, 0.2% gentamycin, and 1% penicillin–streptomycin.

N4X4 cells were preserved in liquid nitrogen in cryovials, and the cryovial was taken and rapidly thawed by swirling the vial delicately using a water bath at 37 °C. Afterward, the thawed cells were centrifuged for 10 min at room temperature at 150 to 200 × g. The supernatant was discarded, and cells were washed with a fresh medium to remove DMSO. The cells were suspended in the medium gently, transferred to the culture vessels, and kept in the incubator. After 80% confluency, cultured media was washed by DMEM, and 800 μL of trypsin was added for detaching the cells from the top of the culture vessels. After watching 90% of cells separated under the microscope, 5 mL DMEM media was added to the vessels and blended using a pipette. Finally, 1 mL of this solution was taken and mixed with 4 mL of DMEM in a new vessel and kept in an incubator for the MTT assay with the samples.

2.3.2. MTT colorimetric assay

The MTT colorimetric assay was employed to assess the cytotoxicity of Triphala. This study was conducted in Dhaka, Bangladesh's Centre for Advanced Research in Sciences (CARS). In a 5% CO_2 environment, N4X4 cells were seeded into 96-well flat-bottom tissue culture plates at a density of 1.5×10^4 cells per well and incubated at 37 °C for 48 h to allow the cells to attach on the surface of the culture plate. Then, the cells were treated with ten μL of sample solution following 24-hour incubation at concentrations of 0.0025–25 mg/mL. The CellTiter 96 non-radioactive cell proliferation assay kit (Promega, USA) was used to assess cytotoxicity after 48-hour incubation. After adding the assay kit, plates were incubated for 4 h. During this incubation period, living cells converted the yellow MTT tetrazolium component of the dye solution to a purple formazan product. Finally, the absorbance was measured at a wavelength of 570 nm using a microplate reader. The absorbance is based on reducing a yellow tetrazolium salt or MTT to purple formazan crystals, and the darker the solution, the greater the number of viable, metabolically active cells. A 2% DMSO solution and cycloheximide played a negative and positive control role, respectively. Two independent experiments were conducted to generate the results, and each experiment was conducted in triplicate. The percentage of cell growth inhibition and IC_{50} was calculated from a concentration–response curve.

$$\%ofcytotoxicity = 100 - \frac{Absorbanceoftestsample}{Absorbanceofnegativecontrol} \times 100$$

2.3.3. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of Triphala extracts

GC-MS analysis of the Triphala extracts was carried out by utilizing a GC-MS-QP 2010 Ultra instrument. An AB innowax column (30 × 0.25 mm id, film thickness 0.25 m) was employed for the analysis. The column oven temperature was initially maintained at 100 °C for 1 min, which was then gradually elevated to 270 °C and maintained for 25 min. The sample injection volume was 0.5 μL for analysis. Helium was employed as the carrier gas at a flow rate of 1.15 mL/min in the splitless mode. The sample injector and detector temperatures were kept at 200 °C and 250 °C, successively, and the split ratio was kept at 200 throughout the experiment. Electrons with an energy of around 70 eV were used in the electron ionization mass spectrometry. In addition to this, for 45 min, mass spectra were finally recorded in the range of 50 *m*/*z* to 650 *m*/*z*.

2.3.4. Identification and quantification of individual phytoconstituents

The individual phytocompounds were detected by matching their retention indices and mass spectra fragmentation patterns to authentic samples available in the Wiley database and data already available in the NIST libraries (Alilou & Akssira, 2021).

Additionally, each component's relative proportion was calculated via measuring and comparing its average peak area to the total peak areas.

2.4. Statistical analysis

Each of the experiments, namely; TPC and TAC, were conducted in triplicates (n = 3), while DPPH was carried out in duplicates (n = 2). MTT colorimetric assay for cytotoxic activity screening was carried out in triplicates, and each experiment was performed in duplicates. Microsoft Office Excel (MS-Excel) 2010 was utilized for all statistical analysis, including mean and standard deviation calculation and graphical representations.

3. Results

3.1. Antioxidant activity of Triphala by DPPH free radical scavenging assay

Triphala's antioxidant effect was evaluated using DPPH free radical scavenging assay for Amalaki, Haritaki, and Bibhitaki individually at concentrations of 3.125 to 1200 μg/mL. The findings exhibited a gradual increment of the percentage inhibition of free radicals via Bibhitaki, Amalaki, Haritaki, and the standard Ascorbic acid. It was observed that concentrations 3.125 to 100 μg/mL (Table 1). However, from 200 to 1200 μg/mL, the standard and samples values remained nearly constant. In this test, the highest concentration of 1200 μg/mL produced the highest percentage of inhibition of free radicals, which was 96.85%, 95.80%, and 96.06% for Amalaki, Bibhitaki, and Haritaki, respectively (Table 1). Moreover, using DPPH test antioxidant effect with 1:1:1 (A:B:H), 1:2:1 (A:B:H), 2:1:1 (A:B:H), and 1:1:2 (A:B:H) ratios of Triphala constituents at concentrations of 3.125 to 100 μg/mL was determined. At the highest concentration of 100 μg/mL, the percentage inhibition of DPPH free radicals was 96.59%, 97.64%, 96.46%, and 95.41% for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H), respectively (Table 2). It is noteworthy to mention that the percentage inhibition of the Ascorbic acid (standard) was 96.98%, and the 2:1:1 (A:B:H) Triphala ratio where Amalaki was double in quantity than Bibhitaki and Haritaki showed more inhibition than that of standard ascorbic acid which was 97.64%. From the perspective of IC_{50} value, 10.29, 15.93, and 12.99 μg/mL of Amalaki, Haritaki, Bibhitaki, respectively, were found to inhibit half of DPPH free radicals effectively. As the IC_{50} value for ascorbic acid was 12.96 μg/mL, Amalaki showed more inhibition at a lower concentration (IC_{50} of Amalaki: 10.29 μg/mL). The IC_{50} values for the Triphala ratios 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H), were 11.28, 7.01, 8.33, and 11.03 μg/mL, respectively and it was observed that all the IC_{50} values were lower compared to the IC_{50} value of Ascorbic acid and the individual components of Triphala as well (Table 3).

3.2. Total phenolic content of Triphala

Total phenolic compounds present in the individual constituents of Triphala as well as at 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H) ratios was measured at different concentrations of 200–1200 μg/mL using TPC test and the findings were expressed in mg of GAE per gram of dried extracts. In the case of the TPC test, the total phenolic content of each component and the mixtures at equivalent and nonequivalent ratios was increased with the increasing concentrations, and Amalaki contained the highest TPC (395.09 mg GAE/g of extract) at its highest concentration of 1200 μg/mL among three fruits (Table 4). Furthermore, at the same concentration, the dried extract showed the highest phenolic content, which was 475.83, 548.9, 510.09, and 491.65 mg of GAE per gram of 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H) ratios, respectively (Table 5).

3.3. Total antioxidant capacity of Triphala

Total antioxidant capacity of individual constituents of Triphala as well as at 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H) ratios was carried out at the concentrations of 200–1200 μg/mL and the findings were expressed in mg of AAE per gram of crude dried extracts. In the case of the TAC test, the total antioxidant capacity of each component and the mixtures at equivalent and nonequivalent ratios was increased with the increasing concentrations, and Haritaki at its highest concentration of 1200 μg/mL had the highest TAC (559.76 mg AAE/g of extract)

Table 1
The % inhibition of DPPH free radical scavenging by Ascorbic acid, Amalaki, Bibhitaki, and Haritaki.

Concentration of Sample (μg/mL)	% inhibition of Ascorbic Acid	% inhibition of Amalaki	% inhibition of Bibhitaki	% inhibition of Haritaki
3.125	20.21	21.52	15.09	19.82
6.25	40.03	42.52	41.34	23.49
12.5	59.06	66.40	63.91	62.86
25	83.07	88.45	82.42	76.64
50	93.05	94.36	93.18	93.70
100	94.88	96.19	94.49	94.88
200	96.19	96.33	95.67	95.01
400	96.19	96.59	95.72	95.67
800	96.85	96.72	95.83	95.93
1200	96.98	96.85	95.84	96.06

Table 2
The % inhibition of DPPH free radical scavenging by different ratios of Triphala constituents.

Concentration of Sample (μg/mL)	% inhibition by 1:1:1 (A:B:H)	% inhibition by 2:1:1 (A:B:H)	% inhibition by 1:2:1 (A:B:H)	% inhibition by 1:1:2 (A:B:H)
3.125	25.98	28.08	17.85	8.92
6.25	44.09	51.44	48.03	53.54
12.5	57.22	65.62	77.30	70.21
25	83.33	86.35	85.70	80.71
50	92.39	93.04	96.06	95.14
100	96.59	97.64	96.46	95.41

Table 3
In DPPH test IC_{50} values of Ascorbic acid, Amalaki, Bibhitaki, Haritaki and different ratios of them.

Samples and Standard	IC_{50} (μg/mL)
Ascorbic acid (Standard)	12.96
Amalaki	10.29
Bibhitaki	12.99
Haritaki	15.93
1:1:1 (A:B:H)	11.28
2:1:1 (A:B:H)	7.01
1:2:1 (A:B:H)	8.33
1:1:2 (A:B:H)	11.03

Table 4
Total phenolic content (TPC) of Amalaki, Bibhitaki and Haritaki is expressed in mg of gallic acid equivalent (GAE)/g of crude extract (CE).

Concentration of Sample (μg/mL)	TPC of Amalaki (mg GAE/g CE)	TPC of Bibhitaki (mg GAE/g CE)	TPC of Haritaki (mg GAE/g CE)
200	178.90	190.66	37.51
400	243.94	210.37	220.66
800	348.23	349.66	254.66
1200	395.09	368.94	260.65

among three fruits (Table 6). Moreover, at the same concentration, the dried extract showed the highest antioxidant capacity, which was 535.83, 675.50, 545.32, and 689.76 mg of ascorbic acid per gram of 1:1:1 (A:B:H), 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H) ratios, respectively (Table 7).

3.4. Cytotoxicity of Triphala

In vitro cytotoxicity test of Triphala at an equivalent ratio of 1:1:1 (A:B:H) and a nonequivalent ratio of 2:1:1 (A:B:H) was assessed using MTT colorimetric assay on N4X4-brain cancer cells. The MTT assay results demonstrated that the % of cell growth inhibition was concentration-dependent at both ratios. The highest toxicity against N4X4 cells was produced by the maximum concentration of 25 mg/mL, which was 78.75% and 95.88% cell growth inhibition for 1:1:1 (A:B:H) and 2:1:1 (A:B:H) ratios, respectively (Table 8, Fig. 3). Fig. 1 and Fig. 2 further exhibited that the number of viable cells reduced with the escalating concentration of extracts at both ratios. IC_{50} values of 1:1:1 (A:B:H) and 2:1:1 (A:B:H) ratios were 15.31 mg/mL and 8.29 mg/mL, respectively.

3.5. Identification and quantification of individual phytoconstituents by GC-MS

GC-MS analysis of individual constituents of Triphala led to the Identification of 17, 18, and 21 compounds in Amalaki, Bibhitaki, and Haritaki extracts, respectively (Figs. 4–6 and Tables 9–11). Predominant compounds were represented by 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (16.969%), Lanosta-7,9(11)-dien-18-oic acid, 22,25-epoxy-3beta,17,20-trihydroxy-, gamma-lactone (19.131%), and 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (10.863%), in Amalaki, Bibhitaki, and Haritaki, respectively. 1,3-Benzenedicarboxylic acid bis(2-ethylhexyl) ester was also found in predominant concentration (12.053%) in the methanol extract of Bibhitaki. Other major com-

Table 5
Total phenolic content (TPC) of Amalaki, Bibhitaki, and Haritaki at different ratios is expressed in mg of gallic acid equivalent (GAE)/g of crude extract (CE).

Concentration of Sample (μg/mL)	TPC of 1:1:1 (A:B:H) (mg GAE/g CE)	TPC of 2:1:1 (A:B:H) (mg GAE/g CE)	TPC of 1:2:1 (A:B:H) (mg GAE/g CE)	TPC of 1:1:2 (A:B:H) (mg GAE/g CE)
200	310.86	390.66	350.90	325.51
400	365.91	430.37	401.94	395.66
800	445.23	510.66	480.23	460.66
1200	475.83	548.94	510.09	491.65

Table 6
Total antioxidant content (TAC) of Triphala is expressed as ascorbic acid equivalent (AAE)/g of crude extract (CE).

Concentration of Sample (μg/mL)	TAC of Amalaki (mg AAE/g CE)	TAC of Bibhitaki (mg AAE/g CE)	TAC) of Haritaki (mg AAE/g CE)
200	16.95	8.51	90.08
400	163.20	121.39	154.76
800	235.95	197.14	417.00
1200	485.50	245.32	559.76

Table 7
Total antioxidant content (TAC) of different ratios of Triphala constituents is expressed as ascorbic acid equivalent (AAE)/g of crude extract (CE).

Concentration of Sample (μg/mL)	TAC of 1:1:1 (A:B:H) (mg AAE/g CE)	TAC of 2:1:1 (A:B:H) (mg AAE/g CE)	TAC of 1:2:1 (A:B:H) (mg AAE/g CE)	TAC of 1:1:2 (A:B:H) (mg AAE/g CE)
200	25.86	37.95	27.51	105.07
400	265.91	293.20	283.39	294.76
800	345.23	385.95	352.14	417.00
1200	535.83	675.50	545.32	689.76

Table 8
The % inhibition of N4X4 cell line in different concentrations along with their IC_{50} values at the 1:1:1(A:B:H) and 2:1:1 (A:B:H) ratios.

Concentration of Sample (mg/mL)	% of Cell Growth Inhibition at 1:1:1 (A:B:H) ratio	% of Cell Growth Inhibition at 2:1:1 (A:B:H) ratio	IC_{50} (mg/mL)	
0.0025	3.96	0.02	1:1:1 (A:B:H)	2:1:1 (A:B:H)
0.025	4.39	12.94		
0.25	5.43	25.93	15.31	8.29
2.5	11.89	54.52		
25	78.88	95.75		

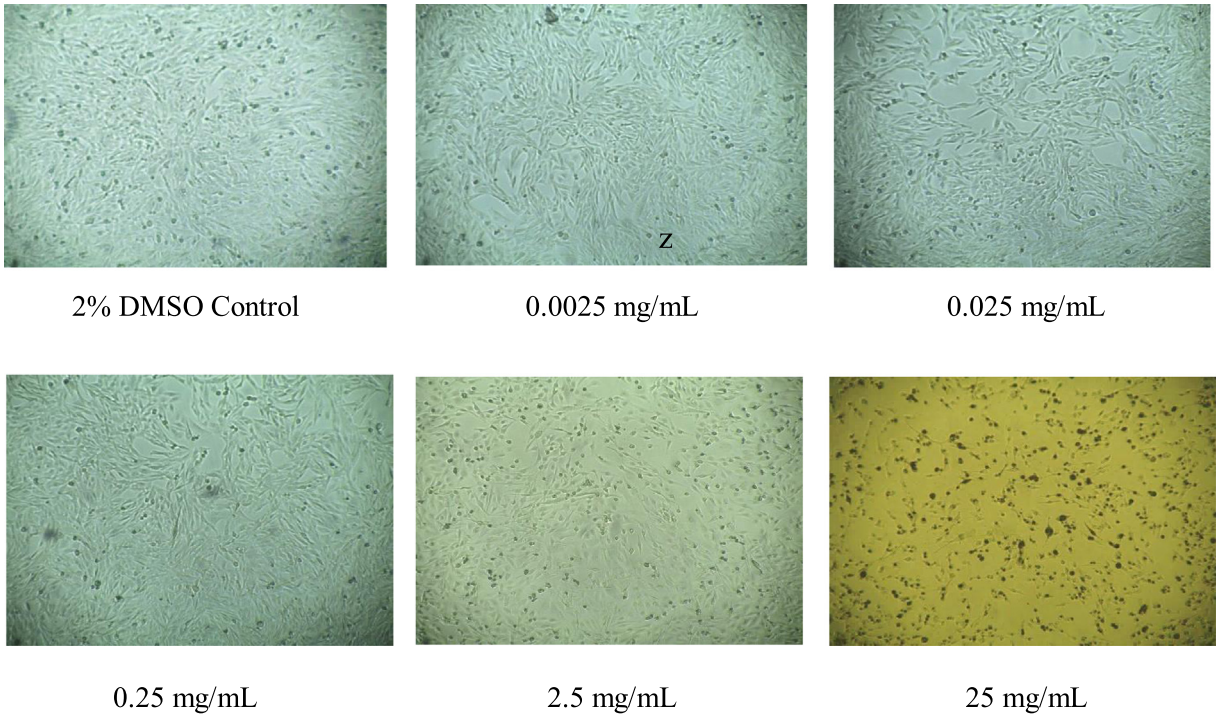

Fig. 1. Cell viability of methanol extract of Triphala, 1:1:1 (A:B:H) ratio, at different extract concentrations of 0.0025 mg/ml, 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, 25 mg/mL and 2% DMSO as control respectively after incubating 48 h in N4X4 cell line.

pounds identified and quantified as 2,4-Dimethylfuran, Trans-2,3-Epoxyoctane, Heptanoic acid, 3-hydroxy-, methyl ester, 4-(2-Hyd roxyethyl)-3-methyl-2-pyrazolin-5-one, 2-Furancarboxylic acid, 2-ethylhexyl ester, paromomycin, octanoic acid in Amalaki extract. Moreover, other principal compounds present in the extract of Bibhitaki were identified and quantified as 2-Cyclopenten-I -one, 5-hydroxy-2,3 –dimethyl, Spirohexane-1-carboxylic acid, ethyl ester, 1-Cyclohexene-1-carboxylic acid, 9-Octadecenoic acid (Z)-, methyl ester, 7-Hexadecenoic acid, methyl ester, (Z)-, 9-Hexadecenoic acid, methyl ester, (Z)-, 2-Cyclopenten-1 -one, 5-hydroxy-2,3-dimethyl, and 2-Pyrrolidinone, 5 -(hydroxymethyl)-. Furthermore, the second most abundant compounds in Haritaki extract were detected as Paromomycin, Furfural, 2-Cyclopenten-1 -one, 5-hydroxy-2,3 –dimethyl-, Carbamic acid, phenyl ester, and 2,3-dihy dro-3,5-dihydroxy-6-methyl-4H-pyran-4-one.

4. Discussion

Reactive oxygen species (ROS) have been implicated in the oxidative damage of cell membrane, cellular protein, DNA which in turn lead to aging and a wide range of ailments, including cancer, cardiovascular diseases, mild cognitive impairment, Alzheimer's disease, Parkinson's disease, and atherosclerosis (Syed Ahmed et al., 2018; Liu et al., 2018; Uddin et al., 2014). Natural antioxidants such as polyphenolic compounds flavonoids can combat these diseases by quenching ROS in the biological system (Pisoschi & Negulescu, 2011).

The antioxidant effect of extracts of Triphala and its individual constituents was assessed by DPPH free radical scavenging assay, TPC, and TAC determination tests. It is noteworthy to mention that our study is reporting the synergistic DPPH free radical scavenging effect, TPC, and TAC of nonequivalent ratios of Triphala components for the first time.

The findings of our study demonstrated that the nonequivalent ratios of Tripahal constituents showed higher synergistic DPPH free radical scavenging, TPC, and TAC than that of equivalent ratios of individual constituents. Furthermore, it is important to mention that Amalaki alone and the nonequivalent ratios of three Triphala constituents where Amalaki was doubled in quantity (2:1:1 = A:B:H), showed the highest synergistic antioxidant potential and the IC_{50} (7.01 μg/mL) of the nonequivalent ratio was much lower than the IC_{50} of the standard antioxidant, ascorbic acid which was 12.96 μg/mL. In our study at 100 μg/mL concentration, Amalaki showed a slightly higher % of free radical inhibition (96.19%) than that of standard ascorbic acid (94.88%), Bibhitaki (94.49%), and Haritaki (94.88%) (Table 1). Our results are supported by previous study findings where Amalaki showed a bit higher DPPH free radical scavenging (94.747%) compared to Bibhitaki (94.406%) and

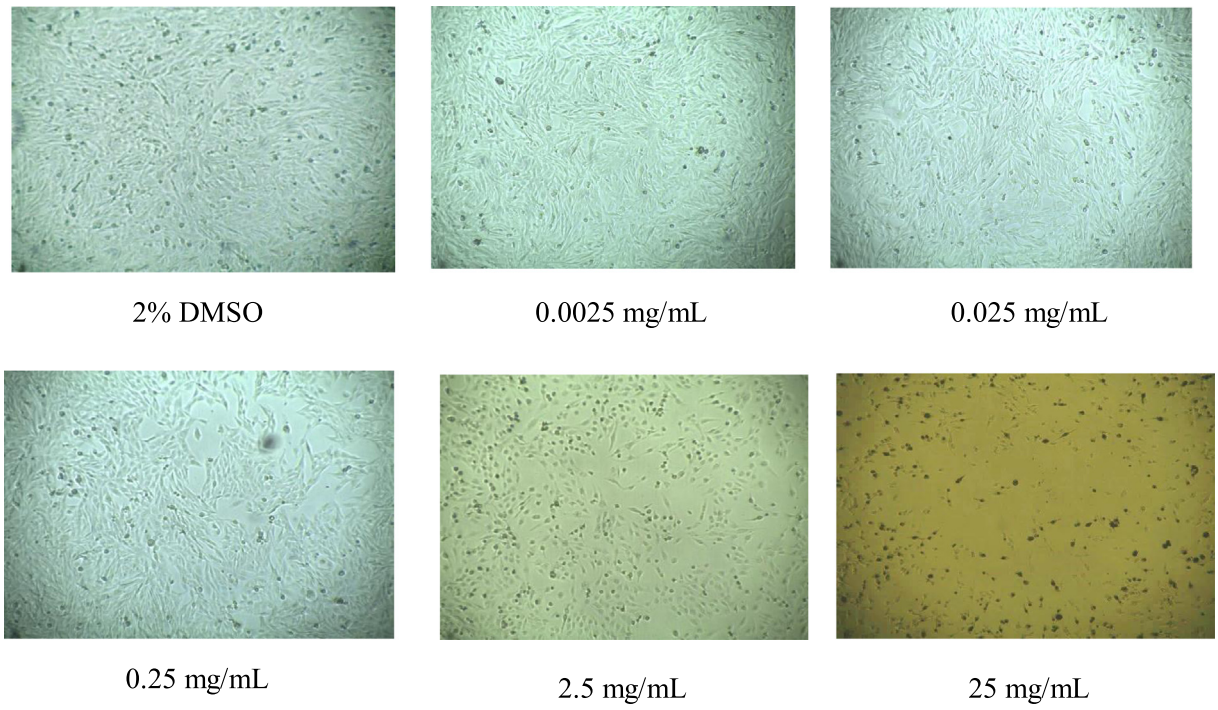

Fig. 2. Cell viability of methanol extract of Triphala, 2:1:1 (A:B:H) ratio, at different extract concentrations of 0.0025 mg/ml, 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, 25 mg/mL and 2% DMSO as control respectively after incubating 48 h in N4X4 cell line.

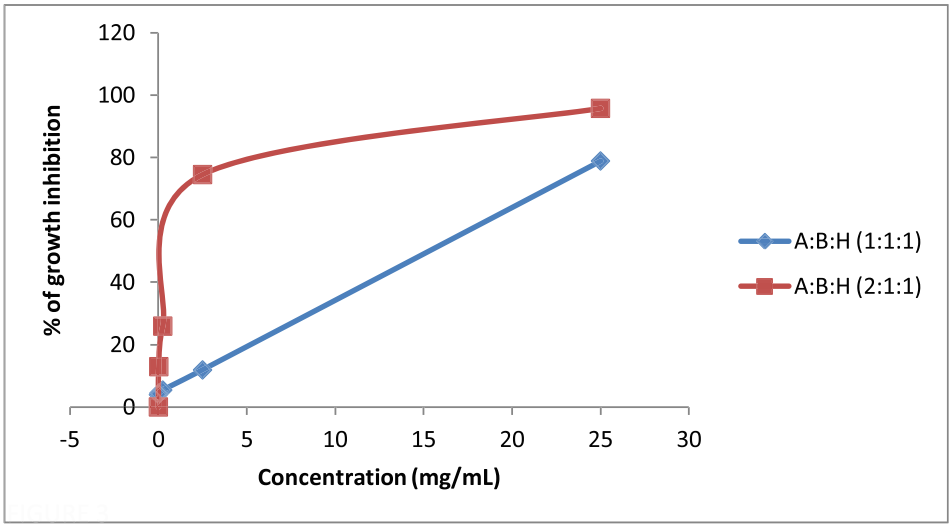

Fig. 3. Cytotoxic effect of methanol crude extracts on N4X4 cell line: The % of cell growth inhibition by equivalent 1:1:1 (A:B:H) and non-equivalent 1:2:1 (A:B:H) ratios of methanol extracts of Triphala constituents.

Haritaki (94.482%) at the concentration of 500 µg/mL (Parveen et al., 2018). However, Amalaki produced only 46.8% inhibition, and ascorbic acid showed 65.6% inhibition of DPPH free radicals at the same concentration (100 µg/mL) in a previous study (Singh et al., 2016). It is important to mention that at concentrations higher than 100 µg/mL DPPH scavenging effect produced was not linear with the increasing concentration ranging from 400 to 1200 µg/mL in the present study (Table 1). In the TPC and TAC assessment, at the concentration of 400 µg/mL, the nonequivalent ratios of Amalaki, Bibhitaki, and Haritaki had significantly higher TPC and TAC than the equivalent ratios of these constituents. The TPC of A:B:H (2:1:1), A:B:H (1:2:1), A:B:H (1:1:2) were found to have 430.37, 401.94, and 395.66 mg GAE/g crude extracts, respectively whereas A:B:H (1:1:1) was found to have 365.91 mg GAE/g crude extracts at the concentration of 400 µg/mL (Table 5). Similarly the TAC of A:B:H (2:1:1), A:B:H (1:2:1), A:B:H (1:1:2) were 293.20, 283.39, and 294.76 mg AAE/g crude extracts, respectively while the TAC of A:B:H (1:1:1) was 265.91 AAE/g crude extract at the same concentration (Table 7). It was evident that the quantity/ratio of Amalaki was a major contributing factor to the DPPH free radical scavenging effect and TPC of Triphala.

This highest antioxidant effect of Amalaki might be attributed to the presence of octadecanoic acid and n-hexadecanoic identified in this extract by our GC-MS analysis, as well as its highest Vit-C content. The fruit juice of Amalaki has been reported to contain

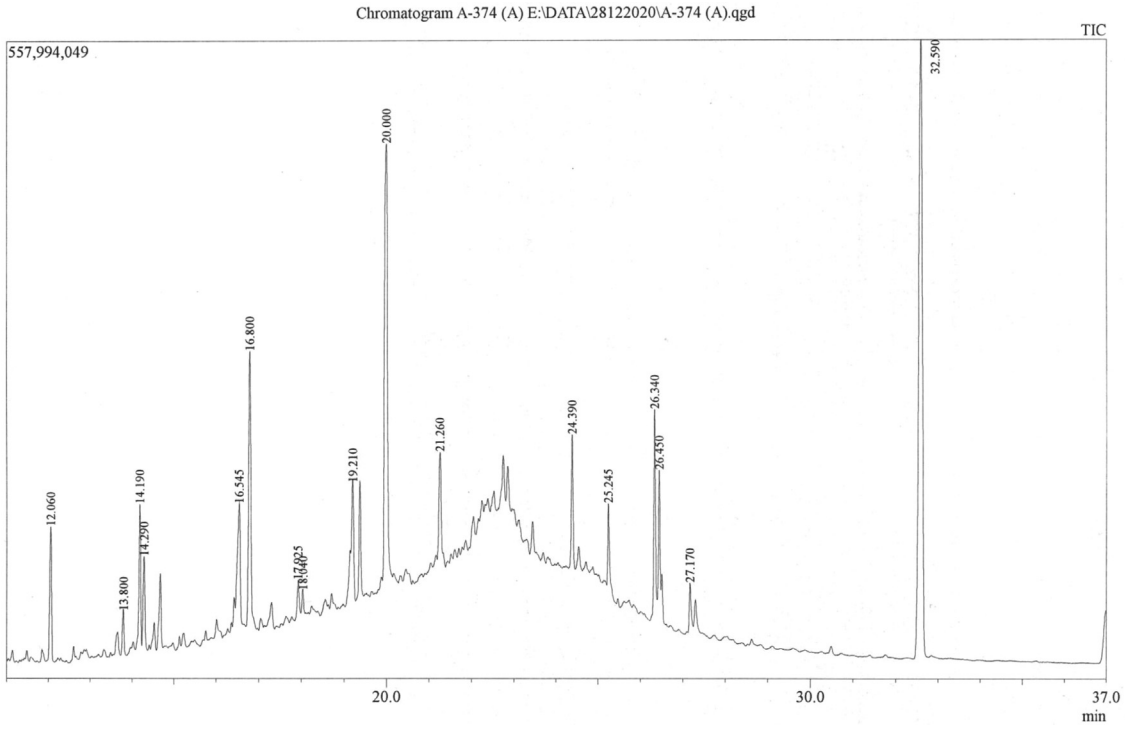

Fig. 4. GC-MS chromatogram of methanol extract of *Phyllanthus emblica* (Amalaki).

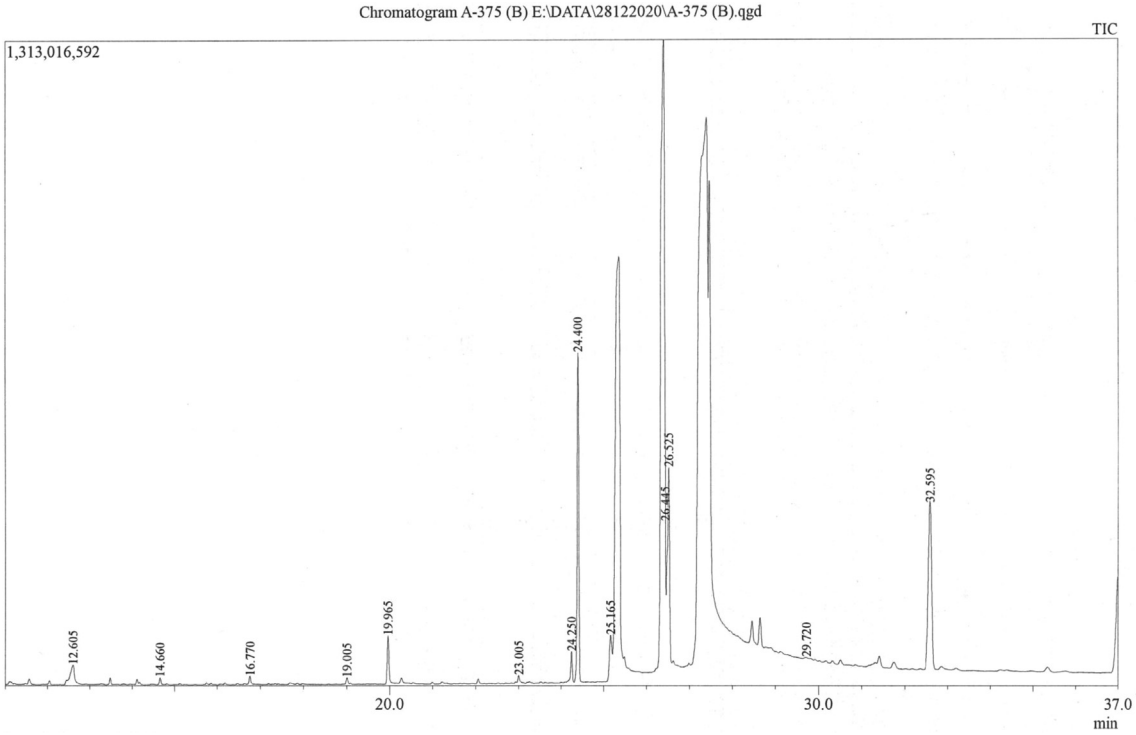

Fig. 5. GC-MS chromatogram of methanol extract of *Terminalia bellirica* (Bibhitaki).

the highest amount of vitamin C (478.56 mg/100 mL) in a previous study (Shastri Brhamashankar & Bhavaprakasha, 1969). This highest TPC of Amalaki could be ascribed to its highest gallic acid content. HPLC analysis of Triphala detected that Amalaki, Bibhitaki, and Haritaki contain 0.081%, 0.005%, and 0.024% w/w of gallic acid, respectively (Pundareekaksha, 2017). Another study reported the TPC of individual fruits of Triphala, and they found that the TPC in terms of gallic acid equivalent varies from 33 to 44% (Naik et al., 2005). Furthermore, the TPC of the crude extracts of Triphala ranging from 195.3 to 296.4 mg of GAE/gm of GAE/gm dry extracts was reported previously (Jayajothi et al., 2004). The findings of a previous study demonstrated the total phenolic content in ethanol

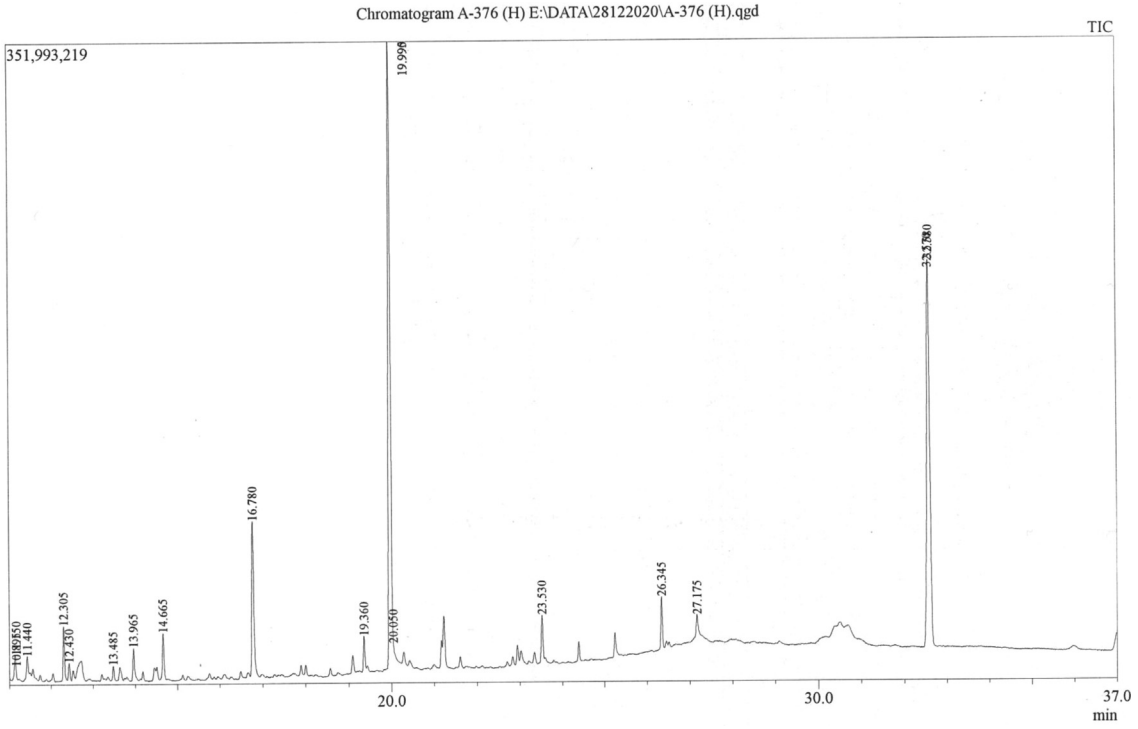

Fig. 6. GC-MS chromatogram of methanol extract of *Terminalia chebula* (Haritaki).

Table 9
Phytochemicals identified and quantified in the methanol extract of Amalaki.

Sl. no	Name of the Compound	Retention time (min)	*m/z*	Area	Height	Conc. (%)
1.	2,4-Dimethylfuran	8.516	95.00	42,100,441	8,365,517	14.936 %
2.	4-(2-Hydroxyethyl)-3-methyl-2-pyrazolin-5-one	10.398	111.00	27,773,063	8,367,038	9.853 %
3.	Trans-2,3-Epoxyoctane	12.058	84.00	39,541,711	8,255,872	14.028 %
4.	2-Furancarboxylic acid, 2-ethylhexyl ester	13.794	95.00	26,873,156	8,204,970	9.534°/o
5.	Heptanoic acid, 3-hydroxy-, methyl ester	14.187	71.00	36,400,612	7,612,806	12.914 %
6.	2-(3-Methylguanidino)ethanol	———	85.00	————	—————	N.D. (Ref) %
7.	d-Glycero-d-*ido*-heptose	————	55.00	—————	—————	N.D. (Ref) %
8.	Paromomycin	16.803	109.00	27,593,590	8,274,656	9.790 %
9.	Octadecanoic acid	17.645	55.00	4,779,887	1,067,771	1.696 %
	Octadecanoic acid	17.926	57.00	9,984,246	2,660,374	3.542 %
10.	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	—————	82.00	—————	—————	N.D. (Ref) %
11.	n-Propyl nonyl ether	—————	69.00	—————	—————	N.D. (Ref) %
12.	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	24.536	60.00	1,885,820	329,949	0.669 %
13.	n-Hexadecanoic acid	—————	55.00	—————	—————	N.D. (Ref) %
14.	Z-(13,14-Epoxy)tetradec-11 -en-1-ol acetate	—————	69.00	—————	—————	N.D. (Ref) %
15.	6,9,12,15-Docosatetraenoic acid, methyl ester	—————	67.00	—————	—————	N.D. (Ref) %
16.	Cyclopentaneundecanoic acid	27.170	55.00	17,104,964	4,791,795	6.068 %
17.	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	32.587	67.00	47,829,702	8,251,805	16.969 %

extract of Triphala was 254 ± 8.3 mg GAE/g of crude extracts (Babu et al., 2013). This highest TAC of Haritaki could be attributed to the compound, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6 methyl- which was detected in the GC-MS analysis of Haritaki extract. This compound has been reported as a strong antioxidant in a previous study (Yu, Zhao, Liu, Zeng, & Hu, 2013) as well as due to its highest ascorbic acid content.

In vitro cytotoxicity test of Triphala at both nonequivalent ratio of 2:1:1 (A:B:H) and equivalent ratio; 1:1:1 (A:B:H) was measured by MTT assay against glioblastoma cells, N4X4. This cancer cell line

Table 10
Phytochemicals identified and quantified in the methanol extract of Bibhitaki.

Sl. no	Name of the Compound	Retention time (min)	*m/z*	Area	Height	Conc. (%)
1.	2-Cyclopenten-I -one, 5-hydroxy-2,3 -dimethyl	19.962	51.00	32,617,077	8,295,690	7.716
2.	Spirohexane-1-carboxylic acid, ethyl ester	19.962	51.00	32,617,077	8,295,690	7.716
3.	1-Cyclohexene-1-carboxylic acid	19.962	51.00	32,617,077	8,295,690	7.716
4.	9-Octadecenoic acid (Z)-, methyl ester	24.250	55.00	22,009,193	7,645,464	5.206
5.	7-Hexadecenoic acid, methyl ester, (Z)-	24.250	55.00	22,009,193	7,645,464	5.206
6.	9-Hexadecenoic acid, methyl ester, (Z)-	24.250	55.00	22,009,193	7,645,464	5.206
7.	Lanosta-7,9(11)-dien-18-oic acid, 22,25-epoxy-3beta,17,20-trihydroxy-, gamma-lactone	26.312	67.00	80,875,411	7,915,536	19.131
8.	Pentaborane(11)	———————	61.00	——————	——————	N.D. (Ref)
9.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6 methyl-	14.659	55.00	7,868,536	2,970,696	1.861
10.	Hydroxy methyl furfural	16.765	97.00	8,661,673	3,536,218	2.049
11.	2-Pyrrolidinone, 5 -(hydroxymethyl)-	19.006	84.00	22,437,120	7,414,633	5.308
12.	2-Cyclopenten-1 -one, 5-hydroxy-2,3-dimethyl	19.962	51.00	32,617,077	8,295,690	7.716
13.	n-Hexadecanoic acid	23.002	60.00	6,849,035	2,557,141	1.620
14.	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	24.402	157.00	17,928,441	8,396,404	4.241
15.	0leicAcid	———————	55.00	——————	——————	N.D. (Ref)
16.	1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester	———————	77.00	——————	——————	N.D. (Ref)
17.	Cyclopentaneundecanoic acid	———————	55.00	——————	——————	N.D. (Ref)
18.	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl	32.598		50,953,031	8,357,116	12.053

Table 11
Phytochemicals identified and quantified in the methanol extract of Haritaki.

Sl. no	Name of the Compound	Retention time (min)	*m/z*	Area	Height	Conc. (%)
1.	1,2,4-Benzenetriol	——————	52.00	——————	——————	N.D. (Ref)
2.	2-Cyclopenten-1 -one, 5-hydroxy-2,3 -dimethyl.	16.776	69.00	32,220,591	8,244,314	6.755
3.	Paromomycin	19.992	57.00	21,209,765	8,108,614	4.446
4.	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl	32.588	167.00	51,817,366	8,382,485	10.863
5.	Carbonocyanidic acid, ethyl ester	7.770	54.00	17,087,391	6,804,018	3.582
6.	Furfural	8.518	95.00	40,835,307	8,388,693	8.561
7.	Cyclohexanone	10.408	55.00	7,084,690	2,567,521	1.485
8.	4-Nonanol	10.891	55.00	8,082,150	3,402,005	1.694
9.	2-Furancarboxaldehyde, 5-methyl-	11.148	53.00	9,810,133	3,950,420	2.057
10.	Cyclobut-1 -enylmethanol	11.435	55.00	10,502,255	3,775,573	2.202
11.	Carbamic acid, phenyl ester	12.303	94.00	19,774,147	7,875,633	4.146
12.	Tetrahydroeyclopenta[1,3]dioxin-4-one	12.431	68.00	15,328,408	5,993,338	3.214
13.	Thymine	13.483	55.00	9,012,657	3,577,835	1.889
14.	2,3-Dimethylfumaric acid	13.964	55.00	12,171,251	4,830,716	2.552
15.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6 methyl-	14.662	55.00	13,752,481	5,271,781	2.883
16.	2-Propenoic acid, 3-phenyl-	19.358	51.00	6,462,997	2,515,742	1.355
17.	Paromomycin	19.993	97.00	41,308,023	8,285,082	8.660
18.	1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)-	23.527	60.00	15,238,417	4,925,917	3.195
19.	Cyclopropanepentanoic acid, 2-undecyl-, methyl-	26.345	55.00	9,445,985	3,855,825	1.980
20.	Cyclopentaneundecanoic acid	——————	55.00	——————	——————	N.D. (Ref)

was selected since no previous study was performed using Triphala extracts and with a hope to find a natural cure for this cancer as glioblastoma is one of the rapidly growing and mostly incurable brain cancers despite advances in cancer treatment modalities (Hanif et al., 2017).

Among three nonequivalent ratios; 2:1:1 (A:B:H), 1:2:1 (A:B:H), and 1:1:2 (A:B:H), the nonequivalent ratio of 2:1:1 (A:B:H) was selected for assessing the cytotoxic effect because of its maximum DPPH radical scavenging potential and TPC estimated in our antioxidant activity study. Moreover, GC-MS-employed characterization of Amalaki extracts divulged the presence of a compound named 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester in the highest concentration of (16.969%). Notably, antitumor/antioxidant activity of the compound has been reported against numer-

ous cancer cell lines, including prostate cancer (PC3), breast cancer (MCF), colon cancer (HCT-116), lung cancer (A549), and pancreatic cancer (MIA PaCa-2) cell lines in a previously conducted study (Save, 2015). Also, Amalaki is more easily edible and contains more gallic acid than Bibhitaki and Haritaki (Pundareekaksha, 2017; Kaur et al., 2005). Importantly, gallic acid is a well-documented polyphenol for its cytotoxic potential (Marienfeld, 2003). Further to mention that gallic acid is one of the key constituents present in Triphala and has the potential to arrest cancer cell proliferation recommending the major factor contributing to the antimutagenic and cytotoxic effects of Triphala (Kaur et al., 2005).

The nonequivalent ratio of Triphala constituents showed significantly higher synergistic cytotoxic activity in N4X4 cells. Thus, the nonequivalent ratio of Triphala composition exhibited a notable difference in synergistic cytotoxic effect produced by 2:1:1 (A:B:H) ratio where the quantity of Amalaki was doubled with an IC_{50} value of 8.29 mg/mL compared to 1:1:1 (A:B:H) ratio with an IC_{50} value of 15.31 mg/mL where Amalaki was in equal quantity as of other two constituents. In the previous study, Triphala showed a strong inhibitory effect on gynecological cancer cells with IC_{50} values ranging 98.28–101.23 μg/mL against three different cancer cells, including ovarian (SKOV-3), cervical (HeLa), and endometrial (HEC-1B) cancer cells. Several studies reported the cytotoxic effect of Triphala where it showed significant cell growth inhibition with an IC_{50} value of 50 μg/mL and induced apoptosis in pancreatic cancer cells (Capan-2) (Prasad & Srivastava, 2020; Shi et al., 2008). Prasad & Srivastava reported the dose-dependent antiproliferative effect of Triphala methanol extract (TME) on human carcinoma stem cells (HCCSC) and colon cancer cells (HCT116). They also reported TME-induced apoptosis properties in HCCSCs (Prasad & Srivastava, 2020). Triphala-induced apoptosis was characterized by enhanced expression of p53 level and Bax (B-cell lymphoma protein 2-associated x)/Bcl-2 (B-cell lymphoma protein 2) ratio via activation of the mitochondrial apoptotic signaling pathway. Since TME exhibited a remarkable anticancer effect against colon cancer cells, it can be taken simultaneously with the conventional chemotherapeutic agents to treat and manage colon cancer (Vadde et al., 2015). Our study also suggested that Triphala has strong cytotoxicity against glioblastoma cells. More importantly, the nonequivalent ratio where the quantity of Amalaki was doubled exhibited significantly higher activity against N4X4-brain cancer cell line than the equivalent ratio of Triphala composition.

In our study, GC-MS analysis of individual constituents of Triphala led to the characterization and quantification of a significant number of compounds belonging to fatty acid, fatty acid ester, alcohol, aminoglycoside, triterpene, etc. in the Amalaki, Bibhitaki, and Haritaki extracts, the components of Triphala. Of those, some compounds have been reported with huge potential to exert antioxidant and cytotoxic effects. Octadecanoic acid, n-Hexadecanoic acid, 1,2,3, Benzenetriol, 9-Octadecenoic acid (Z)-, methyl ester, 9-Hexadecenoic acid, methyl ester, (Z)-, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6 methyl-, Lanosta-7,9(11)-dien-18-oic acid, 22,25-epoxy-3beta,17,20-trihydroxy-, gamma-lactone, and 7-Hexadecenoic acid, methyl ester, (Z)- and have been reported to produce an antioxidant effect (Ganesh, 2017; Lim, Kim, Rhee, & Kim, 2016; Reza et al., 2021; Xia et al., 2014; Yu et al., 2013). 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester and Lanosta-7,9(11)-dien-18-oic acid, 22,25-epoxy-3beta,17,20-trihydroxy-, gamma-lactone have been reported to exert anticancer/antitumor activity (Save, 2015; Xia et al., 2014). More specifically, 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester has been reported with remarkable anticancer activity against PC3, MCF, HCT-116, A549, and MIAPACA cancer cell lines (Save, 2015). Therefore, these GC-MS identified individual compounds along with other polyphenolic compounds and vitamin C might be responsible for the synergistic antioxidant and cytotoxic effect exhibited by the nonequivalent ratios of the Triphala constituents.

5. Conclusion

Our findings revealed the significantly higher synergistic antioxidant activity of mixtures of the individual constituents of Triphala at their nonequivalent ratios than that of an equivalent ratio. It is noteworthy to mention that the nonequivalent ratio at which the quantity of Amalaki was double than other two constituents showed the highest synergistic antioxidant effect. Cytotoxic activity determination on N4X4-brain cancer cells, nonequivalent ratios of individual components of Triphala showed a notable difference in synergistic cytotoxicity compared to an equivalent ratio of them. GC-MS analysis of the three individual constituents of Triphala led to the Identification and quantification of a wide array of compounds. Importantly some of them with antioxidant and cytotoxic potential could be contributing to the exhibited synergistic antioxidant and cytotoxic effects. Thus, it can be inferred that the methanol extract of Triphala at nonequivalent ratios of its three constituents where the quantity of Amalaki is double is expected to be more effective in treating oxidative degenerative diseases and glioblastoma (brain cancer). A new formulation can be developed using this nonequivalent ratio where Amalaki is doubled in quantity, and a clinical trial can be performed to estimate/prove its higher therapeutic benefits.

Funding

This research did not receive any funds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103287.

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