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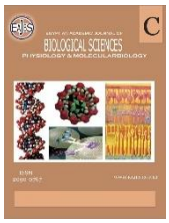
Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 15(2) pp513-527 (2023)*
DOI: 10.21608/EAJBSC.2023.322911

Egypt. Acad. J. Biolog. Sci., 15(2):513-527 (2023)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Assessment of The Antiviral and Antiproliferation Effects of Kombucha Tea

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ARTICLE INFO
Article History
Received:16/9/2023
Accepted:22/10/2023
Available:26/10/2023

Keywords:
Kombucha, HepG-2, Vero cell, anticancer activity, antiviral agent.

ABSTRACT

Kombucha tea (KT) is a traditional non-alcoholic beverage, which is produced from the fermentation of black or green tea with a combination of acetic acid bacteria (AAB) and yeast. In addition to its historical usage in traditional medicine for various ailments, recent studies have demonstrated its potential as anti-cancer and anti-viral agent. Therefore, the objective of this study was to investigate the impact of KT on inducing anti-cancer effects in HepG-2 cells and its potential to exhibit anti-viral activity using Vero cells. MTT assay was conducted to assess the cytotoxicity of the KT and choose the optimal concentration for the study. Three types of cells: Normal human liver cells, HepG-2 cells, and Vero cells treated with KT doses of 10, 25, 50, 100, and 200 mg/ml. The IC_{50} concentration recorded was 54.87 mg/ ml for HEPG-2 tumor cell line and 73.8 mg/ ml for Vero cells. It was demonstrated that the KT had a strong cytotoxic effect on tumor cells as well as an antiproliferative function. However, it exhibited no discernible growth inhibition on normal cell lines. Flow cytometry was employed to study the KT's impact on inducing apoptosis (cell death), by exposing the cells to the KT for different periods (24, 48, 72 hours). It was found that KT has the ability to kill cancer-infected cells compared to the control, in early and late apoptosis of 11.27% and 11.01% respectively. The upregulation of the Bax and P53 genes and the downregulation of the Bcl-2 genes were part of the apoptotic process brought on by KT. These results showed that KT had an antiproliferative effect on HepG-2 cells through apoptosis-mediated cytotoxicity and cell cycle blockage at the G2/M phase. Regarding KT antiviral activity, it had a variable activity against different virus models. The percentage of depletion rate compared to its original viral titer recording was 23.1 %, 9.3% and 8.3% for Hepatitis A virus (HAV), rift valley fever virus (RVFV) and Herpes simplex virus (HSV), respectively. These findings imply that KT might be a good natural product therapy for anti-virus and anti-cancer medicines.

INTRODUCTION

"Kombucha tea" or "tea fugus" is a non-alcoholic beverage traditionally made by fermenting green or black tea using acetic acid bacteria (AAB) and yeast cultures (Sknepnek, *et al.*, 2018).

Kombucha tea (KT) is a very popular, traditional fermented drink consumed throughout the world **(**Sreeramulu *et al.,* 2000). This beverage was originated from Japan (Manchuria) in B.C. 220 (Soares *et al.,* 2021). Following this, it reportedly travelled to Russia and Eastern Europe in the 1800s, then after, during the Second World War, it reportedly made its way to Western Europe and North Africa (Nyhan *et al.,* 2022). Numerous studies have shown that kombucha has an effective therapeutic supplement that prevents cardiovascular disease, helps fight cancer, stimulates the immune system, improves digestive function, and reduces inflammatory problems, also skin problems and hair loss, Meanwhile it conveyed to have like antibiotic, antiviral and antifungal properties in rat lab tests (Lacorn and Hektor 2018, Kapp and Sumner 2019 and Ghandehari *et al.,* 2022). The high organic acid content and hence low pH of kombucha was mostly cited as the cause of its antibacterial properties (Watawana *et al.,* 2015). Gram-positive as well as Gram-negative bacteria such *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes*, and *E. coli* have been shown to be resistant to antimicrobial actions in several investigations (Shahbazi *et al.,* 2018, Vitas *et al.,* 2018, and Zubaidah *et al.,* 2018). Additionally, kombucha has been demonstrated to have antifungal effects on yeasts such *Candida albicans, C. galbrata, C. krusei*, and *A. fumigatus* (Ivanisova *et al.,* 2020 and Nazemi *et al.,* 2019). Furthermore, there is evidence of kombucha processing antiviral capability towards the foot and mouth disease virus (FMDV) (Fu *et al.,* 2015), although no other studies to date have documented the antiviral impact of KT and further research is needed to explore its antiviral effects on other viruses (Nyhan *et al.,* 2022). Gluconic acid, polyphenols, lactic acid, glucuronic acid, vitamins, antibiotics, amino acids, and a range of micronutrients created during fermentation are thought to be responsible for kombucha's health benefits (Rasouli *et al.,* 2021).

Cancer is considered as one of the main causes of death worldwide, thus, it is a serious health problem. The World Health Organization (WHO) reported that nearly 10 million deaths in 2020, or nearly one in six deaths and by 2050, 17.5 million people will die from cancer each year, and 27 million will develop new cancers (Alemi *et al.,* 2018 and WHO 2023).

Medicinal plants' uses have been known for along time, but that use has been over raised in the last 30 years. Nearly, more than 80% of the world population depends on them for some part of primary healthcare (Ekor, 2013, Ahmad *et al.,* 2017 and Ssenku *et al.,* 2022). They are rich in natural products that have biological activities, easy accessibility, cheap and safety (Ahmad *et al.,* 2017). One of the strategies that can help develop anticancer drugs is the induction of apoptosis also known as programmed cell death in tumor cells. Apoptosis plays an essential role in eliminating aberrant and rapidly dividing cells from the body (Atay, *et al.,* 2021 and Morales-Martínez and Vega 2022). As a result, apoptosis inducers have been used in cancer treatment, and the stimulation of apoptosis pathways is a significant way by which chemotherapy medications kill cancer cells. This method is currently recognized as a significant method for evaluating the clinical effectiveness of particular anticancer agents. According to Cho *et al.,* (2005), cell division is the primary regulating mechanism of cellular growth. Cell cycle analysis is a cutting-edge and effective tool for the diagnosis and treatment of cancer. The cell concentration in the sub G1 phase was a sign of apoptosis, whereas the link between the cell cycle and apoptosis is critical in determining how responsive cancer cells to chemotherapy (Haruyo *et al* 2006).

Different tumor suppressor genes, including p53, are implicated in the apoptotic process that is induced by medicinal herbs. By altering the expression of Bcl-2 family members, including Bcl-2 and Bax, either directly or indirectly, the p53 gene causes apoptotic cell death (Reed, 1999 and Levine

et al., 1991). An anti-apoptotic gene called Bcl-2 slows the apoptosis start process by preventing the pro-apoptotic proteins (Youle and Strasser 2008). The p53 gene may affect a cell's sensitivity to apoptosis by upregulating BAX and downregulating BCL-2 (Choi *et al.,* 2000).

The accumulation of nucleic acids, organic acids, and the toxicity of kombucha on the human body during the prolonged duration of fermentation need to be investigated further (Amarasinghe *et al.,* 2018 and Martini 2018). Thus, the objective of the current study is to assess Kambucha's impact on human cell proliferation *in vitro*. By examining the impact of Kambucha on cell cycle arrest, cell apoptosis, and apoptosis-related genes of the HepG-2 cell line, antiproliferation mechanisms as well as these effects were explored. Furthermore, to evaluate the kombucha antiviral activity against Hepatitis A virus (HAV H-10) strain, Rift Valley Fever virus (RVFV) and Herpes Simplex virus type-2 (HSV-2).

MATERIALS AND METHODS

Experimental Design:

Our objective was to investigate the potential anticancer and antiviral effects of KT through *in vitro* experiments.

Kombucha tea (KT) product was kindly supplied from Dr. El Dougdoug, Fac. of Agriculture - Ain Shams University, Cairo, Egypt Extracted metabolite was sterile filtrated using 0.22µm (Millipore-USA).

Cell lines: The selected normal human liver cell, human hepatocellular carcinoma and Vero cell lines were used in this proposal.

HEPG-2 cell line liver hepatocellular carcinoma human cells (Van lJendoorn *et al.,*1997) and were kindly supplied from VACSERA Egypt. Vero cell line, clone CCL-81) is an African green monkey kidney continuous cell line established by (Yasumura and kawakita, 1963).

Viruses: The selected three viruses, Hepatitis A virus (HAV H-10) strain (infectivity titer was 6.5 log (10) / ml). Rift Valley Fever virus (RVFV) Menya –S-258 strain (infectivity titer was 7.5 log (10) / ml.). Herpes simplex virus type - 2 (HSV-2) Egypt infectivity titer was 6 log (10) / ml. were kindly supplied from Dr Aly Fahmy, Head of R&D Egy-VAC, VACSERA Egypt**.**

Evaluation of Cytotoxicity and Cell Proliferation:

In order to assess proliferation, cell viability, and cytotoxicity, the dimethylyhiazolyl tetrazolium bromide (MTT) colorimetric assay (Molecular probes, Eugene, Oregon, USA, Cat. no.V-13154) was employed to quantify cellular metabolic activity. Human normal liver cells (THLE2), hepatocellular carcinoma (HepG-2) and Vero cells were used for evaluation the cytotoxic effect of kombucha tea. Different doses of KT 10, 25, 50, 100, and 200 mg/ml were applied in an incubator with 5% CO2 and 95% humidity. After two gentle washings with ice-cold PBS, each well should have 200 µL of MTT added. In a CO2 incubator, the microplate was incubated at 37 °C for a further 4 hours. The excess medium/MTT was removed, then to dissolve the formazan generated, 100 µL of acidified isopropanol was added to each well. At last, shake the microplate for 15 minutes during incubation. Using a microplate reader, the absorption of each well was assessed at wavelength of 630 nm (ELX800, Biokit, Spain). The findings of the experiment were plotted using sigmoidal and dose-dependent curves. Using this sigmoidal curve, the chemicals dose inhibiting 50% of cells (IC50) was determined.

Quantitative Analysis of Apoptotic Cells Using Flow Cytometry (FC):

Phosphatidylserine (PS) is translocated from the cytoplasmic face to the exterior of the plasma membrane during the early stages of apoptosis when the plasma membrane loses asymmetry. This occurrence is crucial for macrophage recognition of cells suffering apoptosis, enabling the cells to be quickly phagocytosed. The binding of Annexin V-FITC (Annexin V-fluorescein isothiocyanate) to the exposed phosphatidylserine allows fluorometric quantitation of apoptotic cells through the identification of cell surface changes that occur during early stages of apoptosis using

flow cytometry. Late apoptotic and/or necrotic cells may also be identified since the cell membrane lost its selective permeability during late apoptosis, enabling propidium iodide to enter the cells where it bonded to DNA (Annexin V and propidium iodide positive). However, the live cells were not stained. (Arur *et al.*, 2003).

Evaluation of Anticancer Activity Produced by Kombucha:

Anticancer activity of Kombucha extract against HEPG-2 cells was determined according to Petricevich and Mendonça (2003), Cell culture flasks (25 cm^2) containing HEPG-2 cells were supplemented with the IC50 of Kombucha extract, while cells without treatment were left as a negative control.

Determination of Apoptosis-Regulatory Genes the Expression Levels:

According to (Wilfinger *et al.*, 1997), the purity and the concentration of the extracted RNA were assessed by diluting the RNA with D.W. and measuring the optical density via spectrophotometer. RNA purity = A_{260} /A_{280}. The following equation was used to calculate RNA concentration and purity:

Conc. of RNA (µg/ml) = A_{260} X dilution factor X 40

Pure extracted RNA should have an anticipated absorbance range of between 1.7 and 2.1.

cDNA Preparation: Extracted RNA (1 µg), of each sample, arbitrary primer (1 µl) and water that has been treated with DEPC (to 12 µl) were combined, centrifuged for a brief period, and incubated for 5 minutes at 65°C. Samples were set on ice and the following reagents were added to each sample the sequence that follows 4 µl of reaction buffer 5X, 1µl of (20 u/µl) RNase inhibitor (Ribolock), 2µl of dNTP Mix (10 mM), 1µl of (200 u/µl) reverse transcriptase (RvertAid H Minus) and 20 µl Total volume. Samples were carefully combined, spun up, and kept for 5 minutes at 25°C then for 60 min. at 42°C for. The procedure was ended by heating for 5 minutes at 70°C. cDNA (reaction products) was preserved at -70°C.

Quantitative Polymerase Chain Reaction (q-PCR): Using the freshly generated cDNA as templates for PCR, the expression of P53 and Bax (pro-apoptotic) genes and Bcl-2 (anti-apoptotic) gene was assessed. The β-actin gene was applied as a housekeeping gene (internal control gene). Twenty-five µl master mix (dream *Taq* green), 4 µl cDNA. 2 µl of each forward and reverse primer (10 picomole / µl), and 17 µl D.W. nuclease free were pre-denatured for 3 minutes at 94°C. Then the 35 cycles of amplification, the following steps were used: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C for (Bax), 57°C for (P53), and 55°C for (Bcl-2), and extension for 45 seconds at 72°C. By heating for 5 minutes at 72°C, reactions were ended. Sample without adding a template (negative control) was also provided to check for reagent contamination. The results were expressed as 2-ΔΔct (Du *et al., 2012*, and Thangapazham *et al.*, 2007).

Three copies of each polymerase chain reaction were performed. One-way ANOVA was used to determine statistical significance. Table (1) provided information on the primer sequences used.

Table 1: Primer sequences of P53, Bax and Bcl-2 (apoptosis-related) genes and housekeeping gene.

Gene	Primer sequences
P53	F: 5`-TCA GAT CCT AGC GTC GAG CCC-3`
	R: 5`-GGG TGT GGA ATC AAC CCA CAG-3`
Bax	F: 5`-ATG GAC GGG TCC GGG GAG CA-3`
	R: 5`-CCC AGT TGA AGT TGC CGT CA-3`
Bcl-2	F: 5`-GTG AAC TGG GGG AGG ATT GT-3`
	R: 5`-GGA GAA ATC AAA CAG AGG CC-3`
β-actin	F: 5'- AAAACTGGAACGGTGAAGGT -3"
	R: 5'-AACAACGCATCTCATATTTGGAA-3'

Virus Models Seed Stock Preparation:

After discarding the growth medium, 0.5 ml of each virus model used in the present study was added to each 50 cm^3 bottle except that left as a negative control. Bottles were incubated at 37^0C for 1hr. for viral adsorption. Infected TC flasks were periodically rotated for assurance of virus well distribution. The cell monolayer was washed with 5.0 ml PBS approximately to remove excess unabsorbed virus particles and about ml maintenance medium was added. In the case of the control bottle the growth, medium was substituted with a maintenance medium. All bottles were incubated at 37° C and observed daily for virus cytopathic effect (CPE). Repeated freezing (at-70^0C) and thawing for three times to gather the cell-free and cell-associated virus. Cell debris was pelleted by centrifugation at 5,000 rpm. The supernatant containing the virus was transferred carefully and filtered through a 0.45 µm Millipore filter which was previously preconditioned with a growth medium containing 5% FCS to prevent adsorption of the virus to the membrane. Test viruses were amplified by another two successive passages into Vero cell monolayer. The total amplified virus was considered as virus stock that was divided into 1 ml aliquots in sterile screw-capped plastic vials and stored at -70° C. Viral infectivity was measured according to (Reed and Muench, 1938), where test viruses were tenfold diluted in E-199 medium and each dilution was dispensed to a column of 8 wells. Infected plated were incubated for 1-1, 5 hrs for virus adsorption. Inoculation of different virus models was decanted in disinfectant-containing pans. The maintenance medium was dispensed to infected plates and incubated at 37° C for 7 days, with daily microscopic examination for detection the cytopathic effect of the test virus. At the end of the experiment duration, 7 days the tissue culture infective dose (50) for each virus model was determined according to (Reed and Muench, 1938).

Evaluation of Antiviral Activity of KT Extract:

Vero cells were treated in advance with KT extract for 24 h proceeded by viral inoculation to assess the impact of kt extract on the initial phases of viral replication as follows. Vero cells were cultivated in 96-well cell culture plates at a density of 10^5 cells/ml and cultured in 96-well cell culture plates. On confluence, growth media were thrown away. Plates were treated with non-toxic concentrations (100 µl/well) of KT extract 24 h at 37°C with. Meanwhile, control plates were kept and left without treatment for viral control titrationaccording to Petricevich and Mendonça (2003).

Statistical Analysis:

The t-test was used to assess the significance of any differences between groups. P values under 0.05 were regarded as significant. All values in tables were expressed as mean ± SD.

RESULTS

Anti-Proliferous Activity of KT Extract:

MTT assay was carried out to test the KT extract's anticancer efficacy on cell lines. The Mater Plex 2010 program was used to calculate the IC50 and cytotoxicity in a concentration approach utilizing test host cell lines of Normal liver, HepG-2 and Vero cell. The total viability concentration of the normal liver cell was 10 mg/ml (Fig. 1-A), It is well clear that kombucha didn’t have cytotoxic activity on normal cells by increasing the dose. Furthermore, the IC50 concentration was determined to be 54.87 mg/ml for the cancer cell line HepG-2 (Fig. 1-B) and 73.8 mg/ml for the Vero clone (CCL-81) (Fig. 1-C).

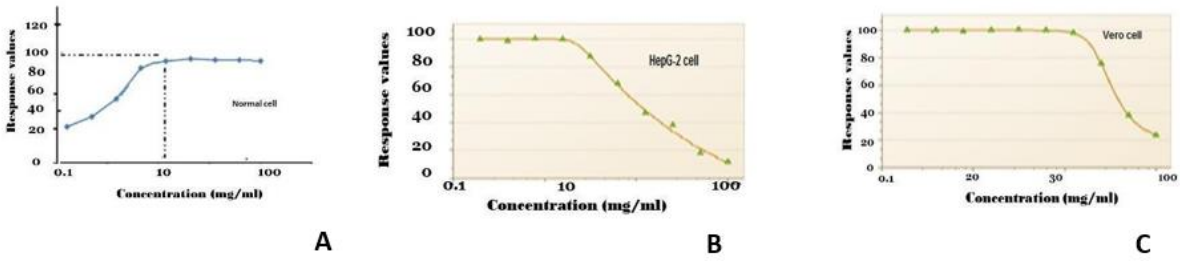

Fig.1: Evaluation of cytotoxicity of Kombucha extract to Normal liver cell line (A), liver cancer cell line (HEPG-2) (B) and normal Vero cell line (C) relative to concentration using Master plex plus 2010 software.

After treating the HepG-2 cell line with KT extract, the degenerative effect was evident, as opposed to untreated HepG-2 cells, which were strongly adherent, uniformly dispersed in the cultivated area, and displayed a polygonal shape with clear boundaries and homogenous cellular contents (Fig. 2-B). However, 24 hours after KT treatment revealed striking morphological aberrations, as cellslost their distinctive shape, rounded out, and detached from the culture surface. Greater cellular abnormalities and wider areas devoid of cells were caused by longer exposure times (Fig. 2-C).Treatment resulted in apparent cellular degeneration, distortion, severe shrinkage, andcondensation (Figs. 2-C& 2-D).

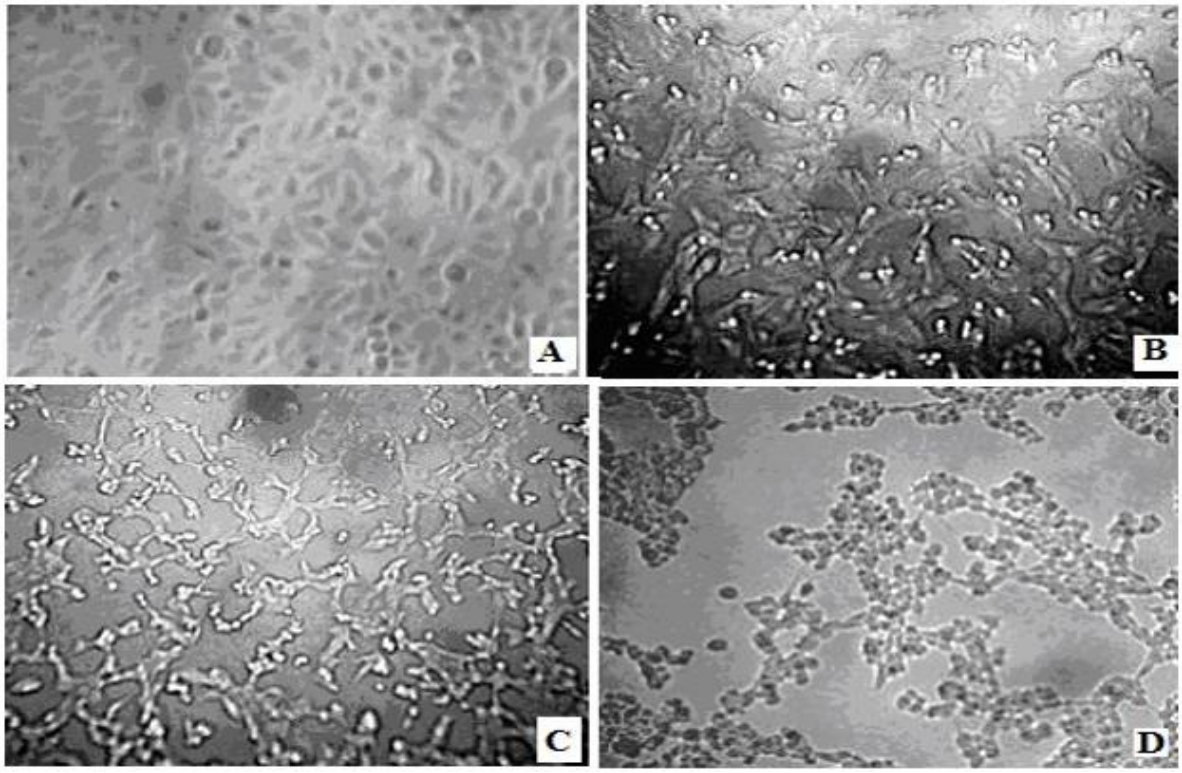

Fig. 2: Evaluation of cytotoxicity of KT to liver cancer cell line (HepG-2) relative to time of ExposureWhere A: Untreated HepG-2 cells, B: Cellular treatment 24h., C: Cellular treatment 48h., D: Cellular treatment 72h.

Evaluation of KT Cytotoxicity:

The inhibition rate of HepG-2 viability by KT was related to cell cycle interception via flow cytometry-based cell cycle dispersion. FC analysis (FACSCalibur) revealed that KT extract could induce

apoptosis in HepG-2 cell line treated relative to the time of exposure. Data were segregated in two-dimensional dot plots, where the zones of early, and late apoptosis as well as surviving cells were segregated depending on luminescence degree. The number of early and late apoptotic cells considerably raised by KT treatment as well as significantly elevated relative to exposure time, while the number of surviving cells was considerably reduced ($P < 0.05$) at 72h post-treatment. As shown in Table (2) & Figs (3 and 4) the apoptotic cell in the 24h treatment was 7.69% versus 0.59% and 3.91% versus 0.34% in the late and early apoptosis, respectively. Moreover, in the 48h. treatment the apoptotic cell was 7.19% versus 0.31% and 7.25% versus 0.52% in the late and early apoptosis, respectively. Meanwhile, in the 72 h. treatment the apoptotic cell was 11.01% versus 0.19% and 11.27% versus 0.71% in the late and early apoptosis, respectively (Table 3) and (Figs 5).

It was found that KT extract at 54.87 mg/ml modified the cell cycle pattern of HepG-2 cells compared with the control group. The G0/G1 phase demonstrated a decrease from 51.74% to 33.89%, 53.29% to 36.31% and 56.92 to 27.52% in the 24h, 48h. and 72 h. treatment, respectively. Interestingly, the S phase proportion decreased partially from 34.16% to 28.14% in the control and KT extract in the 24h treatment respectively, and 25.73% to 22.64% in the control and the treatment of 72h with the KT extract respectively (Table 3) and (Figs. 5).

The proportion of HepG-2 cells at the G2/M phase was raised after KT treated for 24 h (37.97%) versus (14.1%) for the control, moreover in the 48h. treatment was 37.25% versus 19.45% for its control, at least in the 72h. treatment was 49.84% relative to itscontrol 17.35%. These results revealed that KT prevented HepG-2 cells from proliferating via G2/M phase arrest of the cell cycle.

Table 2: Apoptosis stages in HepG-2 cells and control after treatment with kombucha tea for different exposure times.

	Control for 24 h.	**24 h.**	**Control for 48 h.**	**48 h.**	**Control for 72 h.**	**72 h.**
Total cells (Q3)	98.33%	86.48%	98.41%	84.09%	97.66%	75.43%
Early apoptosis (Q4)	0.34%	3.91%	0.52%	7.25%	0.71%	11.27%
Late apoptosis (Q3)	0.59%	7.69%	0.31%	7.19%	0.19%	11.01%

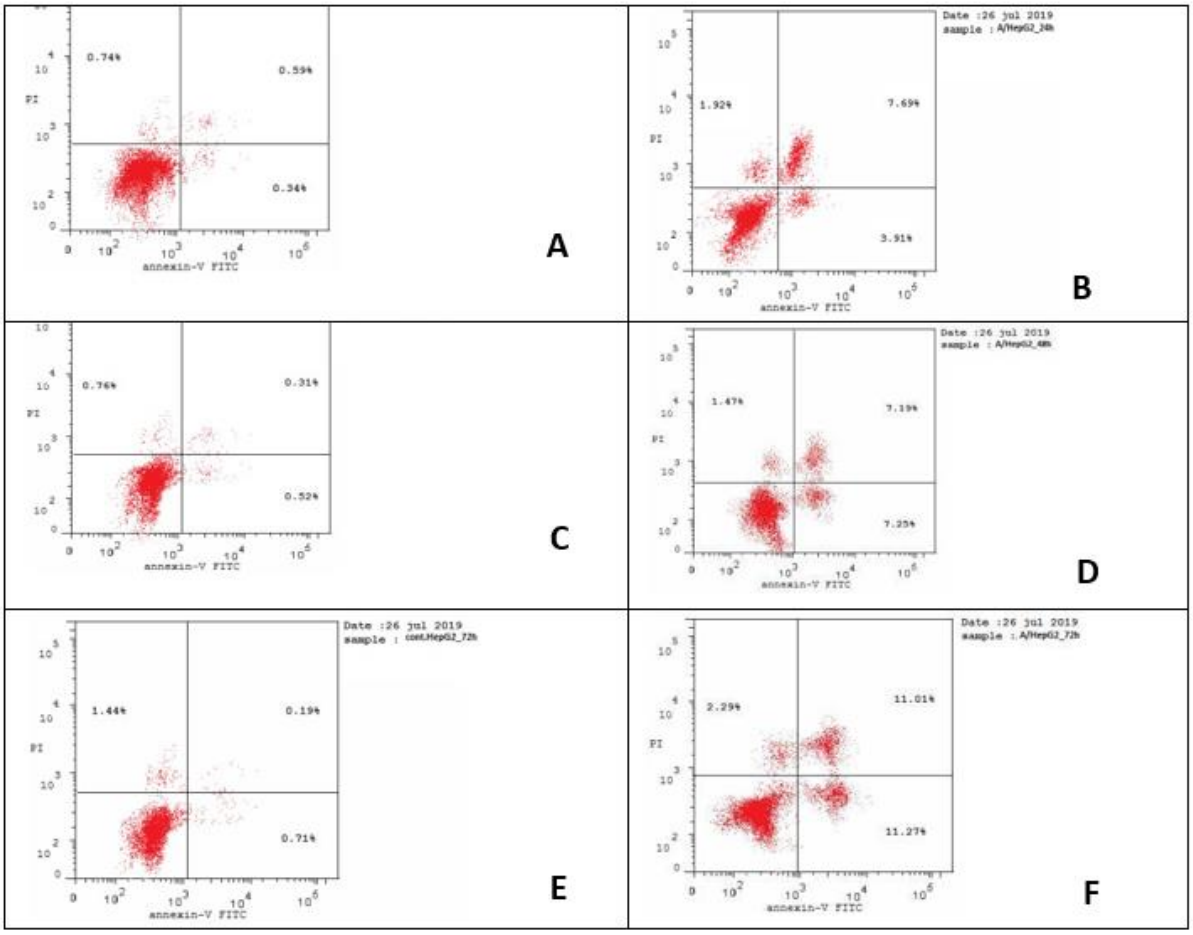

Fig. 3: Apoptosis assay results of HepG-2 cells treated with different exposure times to kombucha tea and their control using flow cytometry.
Where A: Untreated HepG-2 cells 24h., B: 24h. treatment for HepG2 cell, C: Untreated HepG-2 cells 48h. D: 48h. treatment for HepG2 cell, E: Untreated HepG-2 cells 72h. F: 72h. treatment for HepG2 cell.

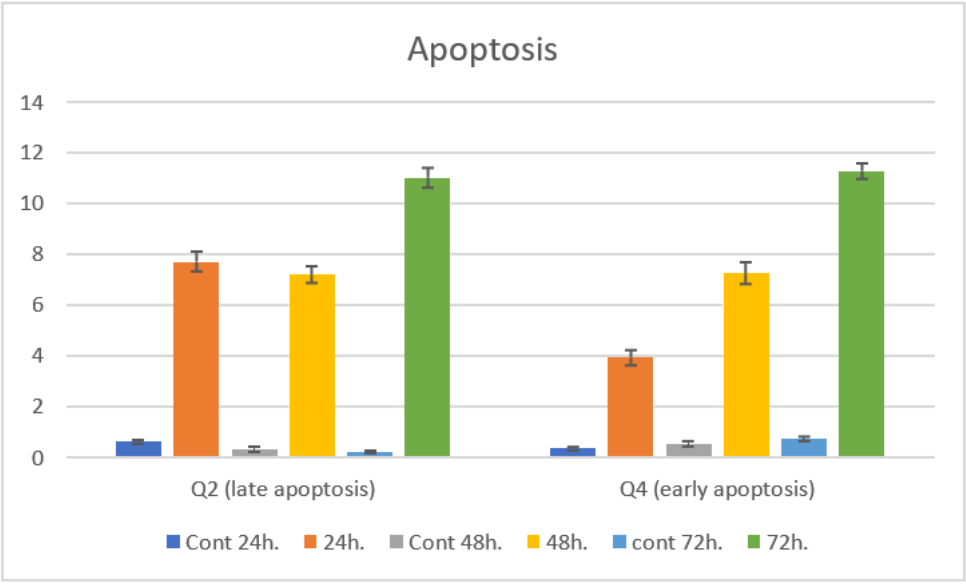

Fig. 4: Evaluation of the early and late apoptotic and necrotic activity of HEPG-2 cell line post-treatment with KT relative to time.

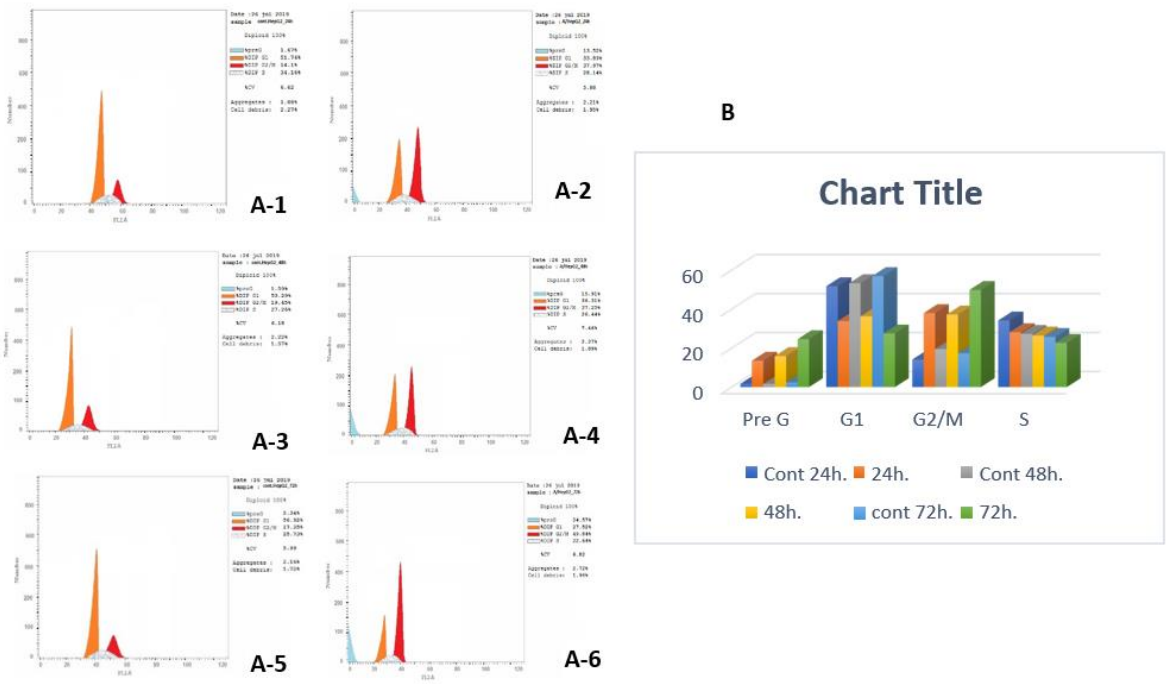

Fig. 5: Propidium iodide (PI) staining was applied to assess the DNA content, and fluorescence was measured and examined. (**A**) Show flow cytometry for control and treated cells. (**B**) Chart shows phases of cell cycle analysis after treatment with different time exposure of kombucha tea (Where A-1: Untreated HepG-2 cells 24h., A-2: 24h. treatment for HepG2 cell, A-3: Untreated HepG-2 cells 48h. A-4: 48h. treatment for HepG2 cell, A-5: Untreated HepG-2 cells 72h. A-6: 72h. treatment for HepG2 cell

Table 3: Arrested cell concentration in each cell cycle after treatment with kombucha with different exposure times (each treatment compared to its control).

	Control for 24 h.	**24 h.**	**Control for 48 h.**	**48 h.**	**Control for 72 h.**	**72 h.**
Pre G	1.67	13.52	1.59	15.91	2.34	24.57
G1	51.74	33.89	53.29	36.31	56.92	27.52
G2/M	14.1	37.97	19.45	37.25	17.35	49.84
S	34.16	28.14	27.26	26.44	25.73	22.64

Effect of KT on Apoptosis-related Genes:

Quantitative real-time PCR (qRT-PCR) was performed to measure the expression level of P53, Bcl-2, and Bax genes (apoptosis-related genes) in HepG-2 cells in order to assess the molecular mechanism of KT extract in HepG-2 cells. The β-actin gene was applied as an internal positive control. Regarding anticancer activity, qRt-PCR was performed and there was clear pre- and anti-apoptotic gene regulation, where P53 and Bax were up-regulated, and BCL-2 was down-regulated compared to control as shown in Figure (6). Therefore, the results indicated that KT extract destroyed HepG-2 cells through an apoptotic process.

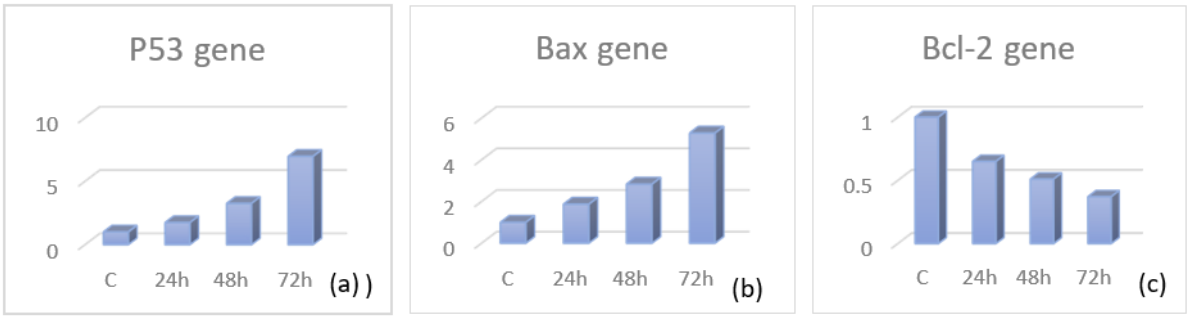

Fig. 6: Detection of anticancer activity of Kombucha to liver cancer cells relative totime by evaluation of the apoptosis gene; P53 (a), Bax (b) and Bcl-2 (c) gene.

Antiviral Activity of KT Extract:

Regarding antiviral activity, it was clear that there was a variable activity against different virus models. The KT extract had partial antiviral activity at the shortest time used and the activity was increased by increasing the time (Fig. 7). Figure (8) shows the infectivity titer % depletion rate compared to the original viral titer recording 1.5 log (10)/ ml [23.1 %] for HAV, 0.7 log (10)/ml [9.3 %] for RVFV and 0.5 log (10) log (10)/ ml [8.3 %] for HSV.

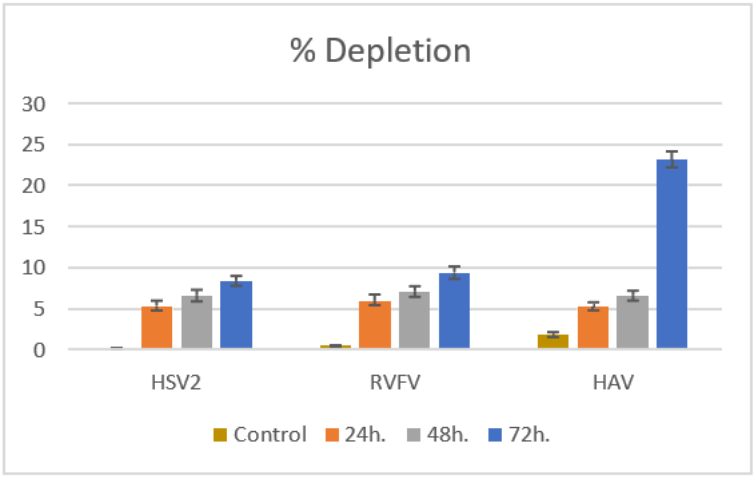

Fig. 7: Evaluation of antiviral activity of KT extract against RNA and DNA virus modelsusing cell culture assay.

DISCUSSION

In conventional therapy, epidemiological studies have shown that green tea has a great protective influence against several kinds of human malignancies. The primary chemical structure components of green tea are catechin polyphenols, which have shown inhibitory effects against the growth of leukemic, colon, lung, and breast cancer cells as well as melanoma and ovarian cancer cells.

Limited references published regarded the biological activity of Kombucha derivatives as antiviral and anticancer agents. The present study investigated the usage of Kombucha (KT) as a therapeutic antivirus and anticancer activity *in vitro* on human cell lines. Our findings revealed that while normal cells may not be affected by KT, cancer cell lines were more severely affected, leading to a reduction in cell viability. Rasouli *et al.*, (2021) evaluated the cytotoxic effect of kombucha on colorectal cancer cells (HCT-116), They discovered a decrease in the viability of human cancer cell lines, therefore, they revealed that KT has cytotoxic properties. Furthermore, they showed that kombucha significantly inhibited the proliferation of HCT-116 *in vitro*. This impact was thought to be due to the rich matrix of kombucha, which

contains polyphenol vitamins, gluconic acid, glucuronic acid, and lactic acid. Moreover, KT had the potential to ameliorate the catechin oxidative activity and cell death in colorectal cancer cell line (HCT-116) more effectively than green tea. This referred to the existenceof acetic acid and gluconic acid in KT.

In the present study, the estimated anticancer activity was estimated by cell cycle analysis in the HEPG-2 cell line subjected to a different exposure time of kombucha and the 72-h. treatment illustrated that the arrested cells increased in the Pre-G and G2/M compared to all the other treatments. As a result, *in vitro* experiments using kombucha showed increased action of anticancer agents that are new strategies against hepatocellular carcinoma.

Apoptosis, a controlled form of cellular death, occurs by both endogenous andexogenous death stimuli, such as tumor necrosis factor-alpha (TNF) and TNF-related apoptosis-inducing ligands, as well as chemotherapeutic drugs (Bai *et al.,* 2012 and Deghrigue *et al.,* 2013).

The p53 proteins, which cause apoptosis, are responsible for the ability of cellular systemsto suppress the development of tumors and the responsiveness to various types of cancer therapy.After cellular stresses, the p53 protein was stabilized and formed a tetramer that attaches to DNA in a specific way, activating a series of apoptosis-associated proteins such as the P53 upregulated modulator of apoptosis, Bax, and Bcl2. Bcl2 proteins, which are found on the mitochondrial membrane, control apoptosis by preserving equilibrium between pro- and anti-apoptotic members.

Using annexin V-FITC to determine the apoptosis activity, staining in HEPG-2 cell line treated with various exposure times of kombucha demonstrated a significant rise in early and lateapoptosis phase when exposed to 72 h. compared to other treatments.

In the present investigation, the mRNA expression levels of P53, Bax and Bcl-2 genes associated with apoptosis, were assessed in HepG-2 cells treated with the KT extract. Our findings illustrated that the KT extract promotes apoptosis, which is triggered by the genes p53, Bax and Bcl-2. This result agreed with numerous studies that revealed the role of p53, Bax and Bcl-2 in initiating apoptosis (Wang *et al.,* 2018, and Rasouli *et al.,* 2021).

Antiviral activity KT extract was clear that a variable activity against different virus models showed the infectivity titer % depletion rate compared to the original viral titer recording 1.8 log (10)/ ml [25.7 %] for HAV, 0.2log (10) log (10)/ ml [3.6 %] for HSV and 0.5 log (10)/ml [7.6 %] for RVFV. Antiviral efficacy was in alignment with Fu *et al.,* (2015) who recorded that antiviral medicine may greatly lessen the severity of an outbreak of genital herpes and reduce the time it takes to heal. Our data was in agreement with reports of *Kombucha America. com* despite the use of different viral models, reported that lactic acid found in Kombucha is effective in inhibiting chicken avian bird flu. Also, *www.Kombuchakamp* reported that kombucha has antiviral and antimicrobial activity. In addition, Ivanisova *et al.,* (2020) reported that kombucha green tea content's phenolic acids can reduce the hepatic toxicity resulting from hepatitis viral infection which may be attributed to the existence of *Usinic acid* that may be effective against several viral models and *lactic acid* is anticancer derivative.

Moreover, antiviral activity may be attributed to the Polysaccharides were reported to havevarious biological properties including antioxidant, antitumor, immuno-modulating, antivirus, reducing blood sugar, etc., (Zubaidah *et al.,* 2018 and Gaggìa, *et al.,* 2019). Studies on kombucha are now mostly concentrated on microbial compositions and interactions (Liu *et al*., 1996; Yang *et al*., 2010; Kumar and Joshi, 2016; *Al-Mohammadi et al., 2021*), antimicrobial protein, antioxidative activity of the fermentation broth (Yuniarto *et al.,* 2016), fermentation conditions (Loncar *et al*., 2006), and so on. There is a lack of research on exo-polysaccharides (EPS) produced by kombucha, which makes it particularly intriguing to explore the submerged cultivation of these EPS using this unique microbial system. Also, data agreed with

Tressa (2009) who recorded that the review provides useful knowledge on the use of medicinal plants to be used in the viruses treatment. Many of these phytochemicals exhibit overlapping and complementary mechanisms of action, which contribute to their antiviral effects. These mechanisms include inhibiting the synthesis of viral RNA or DNA and suppressing viral replication. Various assay methods are employed to evaluate the antiviral activity of these compounds, such as randomized crossover studies, multiple-arm trials, and more compromised designs like nonrandomized crossovers and post- and pre-treatment analyses.

CONCLUSION

According to our current study, kombucha tea exhibits high antiapoptotic activity and even strong antiproliferative effects at the cellular and molecular levels. This indicates a new potential for natural therapy to improve the efficiency of cancer treatment and reduce side effects. The study also highlights the potential of natural product therapy as a promising strategy for cancer treatment. The study showed that kombucha has potential anti-cancer activity against hepatocellular cancer in in vitro models. Further research is needed to develop more efficient and less harmful cancer treatment guidelines using natural products like kombucha. Additionally, our study illustrated that kombucha Tea has antiviral behavior against Herpes simplex virus type - 2 (HSV-2), Rift Valley Fever virus (RVFV), and Hepatitis A virus (HAV H-10). Therefore, it can be used as a natural product therapy as an antiviral.

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