Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(1):695-702. **OPEN ACCESS**

Stigmasterol inhibits proliferation of cancer cells via apoptotic regulatory genes

Atheer Abbas Yaseen Al-Fatlawi

Department of Pharmacology, Faculty of Medicine, Jabir ibn Hayyan Medical University, Najaf, **Iraq.**

***Correspondence:** atheer.alfatlawi@jmu.edu.iq **Accepted:** 12Jan.2019 **Published online:** 12 Mar. 2019

Many natural compounds have a proven role in cancer prevention and treatment. In the present study, we evaluated the antiproliferative effects of commonly consumed phytosterol "stigmasterol" against human cancerous breast (MCF-7) and liver (HepG2) cells and non-cancerous human embryonic kidney (HEK293) cells. The cytotoxicity concentration of stigmasterol against the MCF-7 (IC_{50}; 27.38 µM) and HepG-2 (IC_{50}; 25.80 µM) cells were higher than the HEK29 (IC_{50}; 421.74 µM) cells, as determined by MTT assay. The cytotoxicity result was also confirmed by the LDH assays ($r > 0.983$). The anti-proliferative potential of stigmasterol was also studied at the molecular level. The RT-PCR results showed high expression levels of pro-apoptotic genes (bax, p53), whereas negative expression of anti-apoptotic genes (bcl-2). The stigmasterol treated both cancerous cell lines showed an increase in expression of the gene of caspase-9 and caspase-3. According to the gene expression analysis results, stigmasterol probably activates the apoptosis signaling pathway, and hence genomic DNA fragments were observed through gel electrophoresis. From the results, we concluded that stigmasterol has apoptosis-inducing property and therefore to be evaluated as an anti-cancer therapeutic in the animal model.

Keywords: Stigmasterol, bcl2, bax, p53, caspases

INTRODUCTION

Stigmasterol is a phytosterol and its chemical structure similar to cholesterol (Ryan et al., 2009). It is commonly present in fruits and vegetables and also consumed through different sources like plant oils, plants seeds, soybean, corn, peanuts, cereals, legumes and sunflower oils (Verleyen et al., 2002; Kaur et al., 2011). Stigmasterol and its derivative have been studied for its therapeutic properties. Topical anti-inflammatory effect of stigmasterol reported in 12-O- tetradecanoyl phorbol acetate (TPA) and carrageenan-induced paw oedema model and inhibits cutaneous anaphylaxis (Garcıa et al., 1999; Antwi et al., 2018). Stigmasterol decreased the expression of matrix metalloproteinase and reduced the degradation of cartilage in osteoarthritic rabbits (Gabay et al., 2010). Many researchers have reported beneficial effects of stigmasterol in the treatment of diabetes mellitus (Ramu et al., 2016; Wang et al., 2017). In diabetic rats, it helped to increase secretion of insuline and hepatic glucose metabolism (Ramu et al., 2016). Antioxidant property of stigmasterol evaluated in an animal model and reported activation of hepatic antioxidant enzymes markers viz; alanine transaminase, alkaline phosphatase and aspartate transaminase (Ramu et al., 2016; Ali et al., 2015). In another study, it showed a decrease of hepatic lipid peroxidation and increased of catalase, superoxide dismutase and glutathione enzymes (Panda et al., 2009; Ghosh et al., 2011). Altogether, it provided anti-genotoxic properties of stigmasterol and likely to contribute its chemo preventive properties (Ramu et al., 2016). Anti-tumor activity of stigmasterol also reported in

Ehrlich Ascites Carcinoma in mice (Ghosh et al., 2011), whereas antiproliferative and toxicity reported in human vascular smooth muscle cells A7R5 (Li et al., 2015), hepatoma cells HepG2 (Kim et al., 2014), and human monocyte cell line U937 (Yvonne et al., 2010).

The information on its antiproliferative properties and apoptotic mechanism remain limited. Therefore, in the current study, antiproliferative effect of stigmasterol was evaluated in human breast (MCF-7) and hepatic (HepG2) cancerous cells. To confirm of apoptosis, we also studied the expression level of apoptotic regulatory genes.

MATERIALS AND METHODS

Chemicals:

Stigmasterol compound was procured from the Sigma-Aldrich (USA). The medium of the tissue culture obtained from Hi-Media Company (from India). All solvents and reagents were purchaged from Merck Company (from India).

Cells culture condition:

Human hepatocellular carcinoma (HepG2), non-cancerous human embryonic kidney (HEK293), and human breast cancer (MCF-7) cell lines were obtained from Cell Sciences National Centre (India). The cells were cultured in DMEM (Dulbecco's Modified Eagle) (Irshad et al., 2014). The culture was grown at 37°C and 5% CO_2-incubator in a humidified environmental. All the experiments were performed in the same condition.

Cytotoxicity assay:

The activity of the cellular metabolic of cells was examined by MTT assay. About 2x 10^4 cells inoculated into the each wells of 96 well plate and kept in CO_2-incubator for 24 hour for attaching the cell at well bottom. For treatment, stock solution of stigmasterol was prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium to obtained final concentration of DMSO less than 0.03%. Stigmasterol (15- 50 µM) was treated for 24 and 48 hour, respectively. After the treatment duration, MTT assay was performed according to standardized method (Irshad et al., 2014). The optical density (OD) was measured at 570 nanometer by using Microplate Reader device (Bio-Rad, USA). The live cells metabolic activity was calculated by the equation describe by Irshad et al., (2014).

The total means (± standard deviation) cells metabolic activity was determined of the stigmasterol level, and the better cell was utilized to determining IC_{50} value.

LDH enzyme technique:

LDH enzyme leaked into the culture medium after the cell membrane destruction. The standardized protocol was used for LDH enzyme assay (Al-Fatlawi et al., 2014). After the treatment with stigmasterol (15-50 µM), LDH enzyme in culture medium was determined by kit called Cytoscan™-LDH assay kit (G-Biosciences, USA). Microplate Reader (Bio-Rad, USA) was used for reading final reaction at 490 nanometer. The LDH results were correlated with the MTT assay.

RT-PCR:

The mRNA expression level of apoptosis genes (bax, bcl-2, p53, caspase-9 and caspase-3 genes) were determined by pre-standardized method after the treatment of stigmasterol for 48 hours (Al-Fatlawi et al., 2014). Total RNA of treated and untreated cells were isolated by commercially available kit (Invitrogen). The extracted RNA (2 µL) was converted into the cDNA by using commercially available RevertAid™ first Kit (Fermentas Life Science, USA). Finally, cDNA was used as template for amplification of each gene using specific primers (Table 1). A house keeping gene (GADH) was used as an internal marker. The final products each gene was visualized by electrophoresis in agarose gel (2%) and Gel-Doc system was used for photography (Bio-Rad, USA).

Table 1: Oligonucleotide primers.

Genes	Primer sequence
p53	F- 5' CCAGCAGCTCCTACACCGGC 3'
	R- 5' GAAACCGTAGCTGCCCTG 3'
Bcl-2	F- 5' GGTCGCCAGGACCTCGCCGC 3'
	R- 5' AGTCGTCGCCGGCCTGGCG 3'
Bax	F- 5' GAGCTGCAGAGGATGATTGC 3
	R- 5'CCGGGAGCGGCTGTTGGGCT 3'
Casp-3	F- 5' GTACAGATGTCGATGCAGC 3'
	R- 5' CACAATTTCTTCACGTGTA 3'
Casp-9	F- 5' CCTGCGGCGGTGCCGGCTGC 3'
	R-5' GTGTCCTCTAAGCAGGAGAT 3'
GAPDH	F- 5' GTGATGGGATTTCCATTGAT 3'
	R- 5' GGAGTCAACGGATTTGGT 3'

DNA fragmentation technique:

A pre-standardized method used for fragmentation study (Al-Fatlawi et al., 2015). Stigmasterol (IC_{50}; for 48 hours) treated cells were collected and washed with PBS. The fragmented DNA was extracted by commercially available DNA ladder kit (G-Biosciences, USA). The fragmented DNA was separated in agarose gel (1.8%). The photograph was recorded by using a Gel-Doc system (USA, Bio-Rad Company).

Statistical tests:

ANOVA test is used to analysis of the statistical significance of the experimental, wherever the mean ± standard error was used for determining from triplicate samples for all groups.

RESULTS AND DISCUSSION:

Cytotoxicity:

MTT assay was used for the screening of proliferation and viability of cells at the different dose of stigmasterol. Purple formazan is formed by active cells due to reduction of MTT salt by the mitochondrial succinate dehydrogenase enzyme (Irshad et al., 2014). The result showed decrease of viability of cancerous MCF-7 and HepG2 cells with increase of treatment duration and dose of stigmaterol ($p < 0.005$) (Fig. 1). The IC_{50} of stigmasterol in MCF-7 and HepG2 cells after 48 hour was 27.38 ± 0.21 and 25.80 ± 0.73 µM respectively ($p < 0.05$). At IC_{50} dose, MCF-7 and HepG2 cells showed cytotoxicity by 34.65 and 24.45% after 24 hours treatment, respectively. The cytotoxicity determined in MTT assay was correlated with the LDH enzyme leakage at both time points (24 and 48 hours; $r > 0.983$). The results obtained from both assays provided evidence of stigmasterol has cytotoxicity against cancerous cells. However, both assays work on different principals (Al-Fatlawi et al., 2015).

The cytotoxicity concentration of stigmasterol was also evaluated against non-cancer human embryonic kidney cells (HEK-239). IC_{50} value of stigmasterol against HEK-239 was higher than the cancerous cells (i.e., 421.74 ± 0.48 µM). In a study IC_{50} value of stigmasterol (isolated from a plant, *Kopsia singapurensis* Ridl.) against the MCF-7 cells was 14.5µg/mL (Shan et al., 2014). In another study cytotoxicity of stigmasterol (isolated from *Navicula incerta*) against HepG2 cells was 54% at a concentration of 20 µM (Kim et al., 2014). Similarly, stigmasterol isolated from the *Premna serratifolia* Linn. and reported cytotoxicity against human small cell lung carcinoma (A-549; IC_{50}-82.76 PPM), hepatic cancer (HepG-2; IC_{50}-74.29 PPM) and rat skeletal muscle non-cancerous cell (L-6; IC_{50}- 160.2 PPM) (Biradi and Hullatti, 2017). From the results, we concluded that stigmasterol showed high cytotoxicity potential against the cancerous cells in compared to the non-cancerous cells.

Expression levels of bcl-2 and p53, bax genes:

Reverse transcriptase-PCR used to study of genes (p53 and bcl-2, bax) in HepG2 and MCF-7 cells, which are generally involved in apoptosis and cellular proliferation (Basu and Haldar, 1998). The change in mRNA expression was compared by housekeeping gene. In fig. 2-4, densitometry study indicated relative band intensity of mRNA on the gel (TotalLab Quant). Cells incubated with stigmasterol showed dose-dependent increased level of mRNA transcripts of tumor suppressor p53 gene in both cell lines (Fig. 2,3). At IC_{50} level of stigmasterol, p53 mRNA relative band intensity displayed an increase of 2.2 fold in MCF-7 and 2.4 fold in HepG2 cells, as compared to the untreated control.

p53 gene is a negative regulator of bcl-2 gene and positive regulatory of the bax gene (Basu and Haldar, 1998; Irshad et al., 2015). In this study, the bax gene expression level was not visible in untreated cells. However, stigmasterol treated MCF-7 and HepG2 cells also up-regulation of bax gene and down-regulation of bcl-2 gene in a dose-dependent manner ($p < 0.05$). The result showed the expression ration of bax/bcl-2 increased in both cell lines with the increase of stigmasterol concentration. The result was consistent with a previous study in which, stigmasterol induce the expression of p53 gene and bax gene but decrease expression bcl-2 gene in HepG2 cell line (Kim et al., 2014). From the result, we concluded that the increased ratio of bax/bcl-2 gene promotes the apoptosis process in stigmasterol treated cancerous cells (Kastan et al., 2004).

Caspase genes expression:

Caspase is a marker in apoptotic signalling which detected the apoptosis in stigmasterol treated HepG2 and MCF-7 cells. The result showed that the stigmasterol treated both cell lines exhibited mRNA expression of caspase-9 and -3 genes (Fig. 5-6). At IC_{50} value of stigmasterol, caspase genes expression was increased several times as compared with the untreated group in both cell lines ($p= 0.007$-0.015). The translational product of caspase-9 gene has been reported to initiates the cascade of

apoptosis.[23] However, caspase-3 has a great role in apoptosis (the final phase) (Slee et al., 2001; Parrish et al., 2013). Our result was supported by a study in which stigmasterol up-regulated the expression of caspase-8 and 9 in MCF-7 cancerous cells (Kim et al., 2014). Genomic DNA fragmentation DNA fragments are referred to the clear hallmark of apoptosis (Wong et al., 2011). **In fig. 7,** stigmasterol treated cancerous cells clearly showed ladder pattern genomic DNA in the gels, in compared to the untreated control.

The result concluded that the stigmasterol activates an apoptotic process in which the genomic DNA is cleaved into fragments by the endogenous endonucleases.

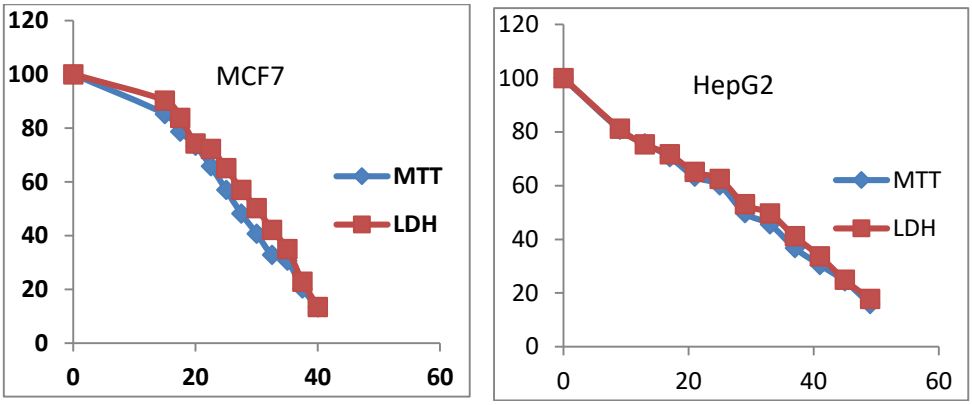

Figure 1: Cytotoxicity trend of stigmasterol against MCF-7 and HepG2 cancerous cells.

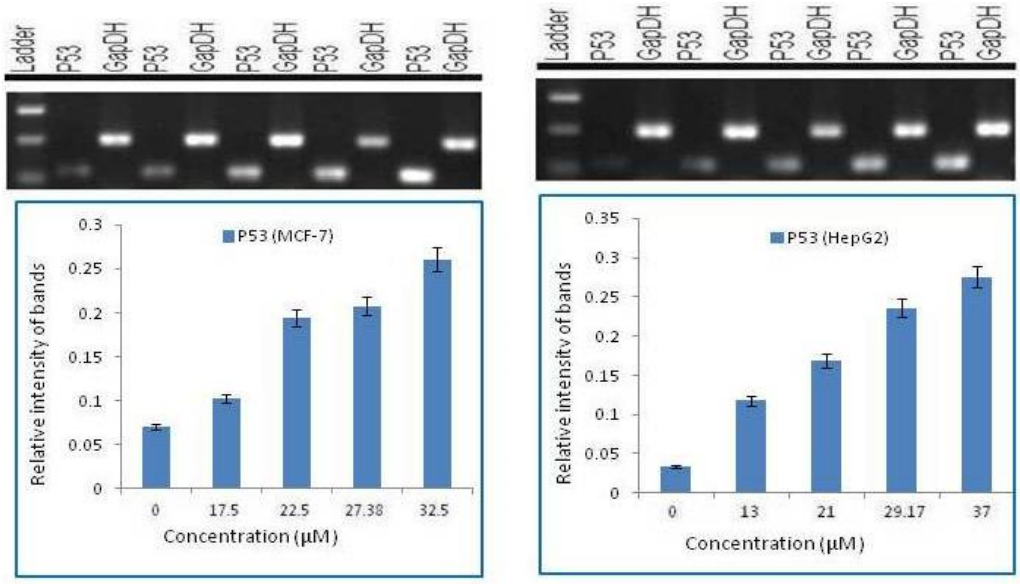

Figure 2: Up-expression of p53 gene with the increase of stigmasterol concentration. Bands intensity was observed by densitometry technique.

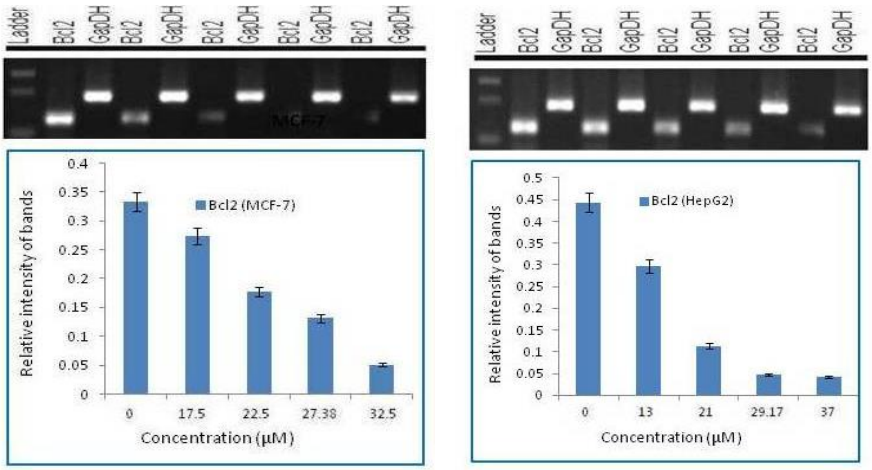

Figure 3: Lower-expression of bcl-2 gene with the increase of stigmasterol concentration. Bands intensity was observed by densitometry technique.

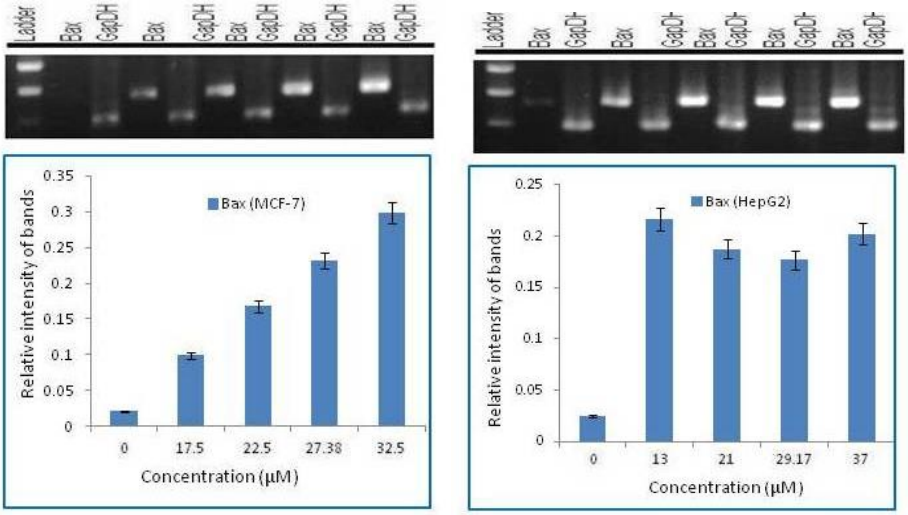

Figure 4: Up-expression of bax gene with the increase of stigmasterol concentration. Bands intensity was observed by densitometry technique.

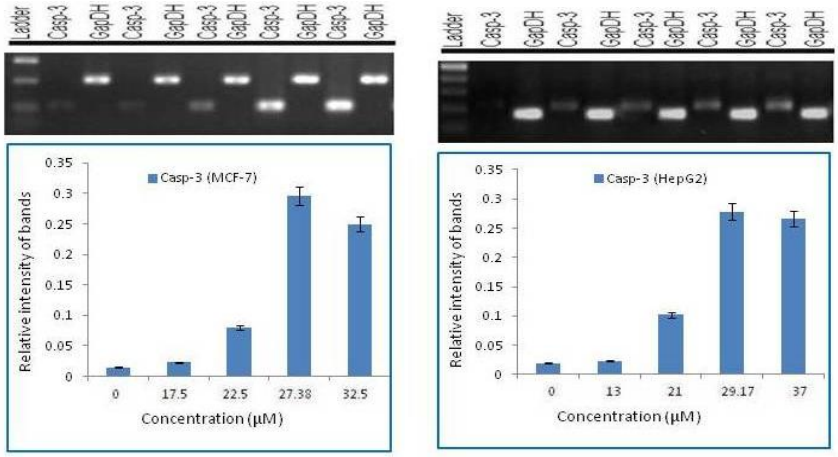

Figure 5: Expression of caspase-3 gene in stigmasterol treated cells. Bands intensity was observed by densitometry technique.

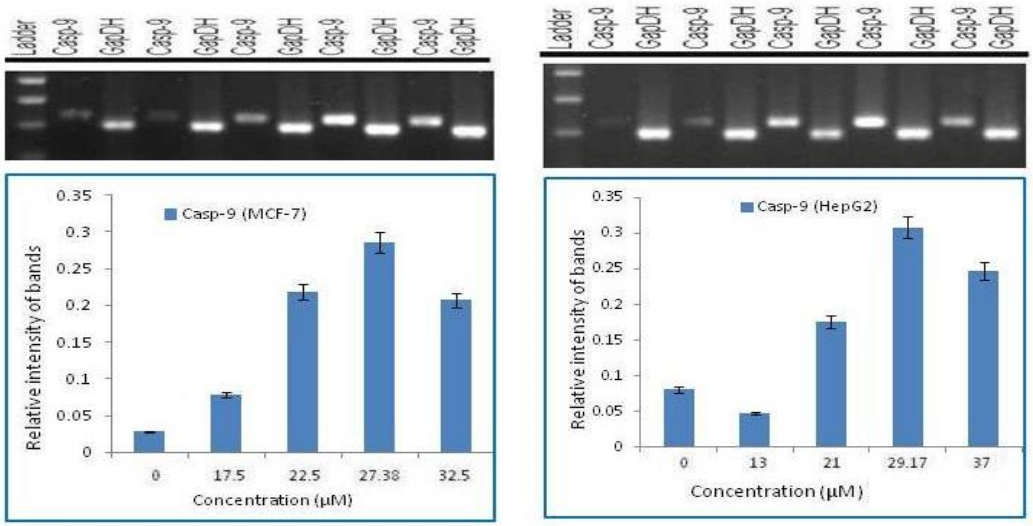

Figure 6: Expression of caspase-9 gene in stigmasterol treated cells. Bands intensity was observed by densitometry technique.

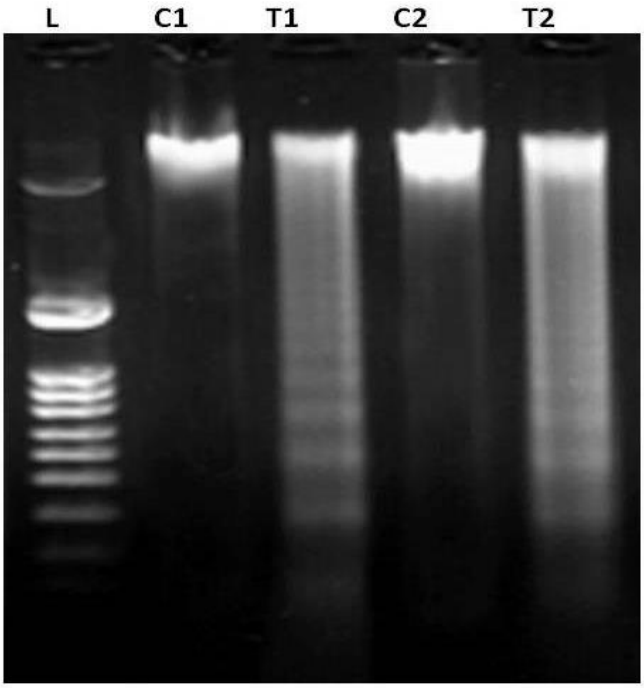

Figure 7: Genomic DNA fragmentation (L, ladder; C1, control MCF-7; T1, treated MCF-7; C1, control HepG-2; and T2, treated HepG-2).

The results were consistent with other study, in which phytosterols have an inductive effect of apoptosis in breast cancer, prostate cancer and human colon cancer cells (Awad and Fink, 2000; Woyengo et al., 2009; Grattan et al., 2013).

CONCLUSION

Our findings provide evidence that stigmasterol exerts anti-proliferative activities against the breast and liver cancer cells. They also exhibited little impact on the viability of normal non-cancerous human embryonic kidney cells in compared to the cancerous cells. This study also found the stigmasterol helped to regulate apoptotic regulatory genes in cancerous cells. Therefore, this compound may be beneficial therapy without possible side effects on normal cells.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

Author acknowledged the Department of Pharmacology, Faculty of Medicine, Jabir ibn Hayyan Medical University, Najaf, Iraq for the laboratory support.

FUNDING: None

AUTHOR CONTRIBUTIONS

AAYA designed and performleed the experiments and also wrote the manuscript. Author read and approved the final version of manuscript.

REFERENCES

Al-Fatlawi A. A., Al-Fatlawi A. A. & Irshad M., & Rahisuddin. 2015. Effect of parthenolide on growth and apoptosis regulatory genes of human cancer cell lines. Pharmaceutical Biology,53(1), 104-109.

Al-Fatlawi A. A., Rahisuddin. & Ahmad A. 2014. Cytotoxicity and pro-apoptotic activity of carvacrol on human breast cancer cell line MCF-7. World Journal of Pharmaceutical Sciences. 2(10), 1218-1223.

Ali H., Dixit S., Ali D., Alqahtani S. M., Alkahtani S., & Alarifi, S. 2015. Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. Drug Design, Development & Therapy, 9, 2793.

Antwi A. O., Obiri D. D., Osafo N., Essel L. B., Forkuo A. D., & Atobiga, C. 2018. Stigmasterol Alleviates Cutaneous Allergic Responses in Rodents. BioMed Research International, 2018.

Awad A. B., & Fink C. S. 2000. Phytosterols as anticancer dietary components: evidence and mechanism of action. The Journal of Nutrition, 130(9), 2127-2130.

Basu A., & Haldar S. 1998. The relationship between BcI2, Bax and p53: consequences for cell cycle progression and cell death. Molecular Human Reproduction, 4(12), 1099-1109.

Biradi M. & Hullatti K. 2017. Bioactivity guided isolation of cytotoxic terpenoids and steroids from Premna serratifolia. Pharmaceutical Biology, 55(1), 1375-1379.

Gabay O., Sanchez C., Salvat C., Chevy F., Breton M., Nourissat, G. & Berenbaum F. 2010. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. Osteoarthritis & Cartilage, 18(1), 106-116.

Garcia M. D., Saenz M. T., Gomez M. A., & Fernandez M. A.1999. Topical antiinflammatory activity of phytosterols isolated from Eryngium foetidum on chronic and acute inflammation models. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 13(1), 78-80.

Ghosh T., Maity T. K., & Singh J. 2011. Evaluation of antitumor activity of stigmasterol, a constituent isolated from Bacopa monnieri Linn aerial parts against Ehrlich Ascites Carcinoma in mice. Oriental Pharmacy & Experimental Medicine, 11(1), 41-49.

Grattan B. 2013. Plant sterols as anticancer nutrients: evidence for their role in breast cancer. Nutrients, 5(2), 359-387.

Irshad M., Mandal R. K., Al-Drees A., Khalil M. S., & Abdulghani H. M. 2015. No Evidence of Association of the Arg72Pro p53 Gene Polymorphism with Cancer Risk in the Saudi

Population: a Meta-Analysis. Asian Pacific Journal of Cancer Prevention, 16(14), 5663-5667.

Irshad M., Mehdi S. J., Al-Fatlawi A. A., Zafaryab M., Ali A., Ahmad I. & Rizvi M. M. A. 2014. Phytochemical composition of Cassia fistula fruit extracts and its anticancer activity against human cancer cell lines. Journal of Biologically Active Products from Nature, 4(3), 158-170.

Kastan M. B., & Bartek J. 2004. Cell-cycle checkpoints and cancer. Nature, 432(7015), 316.

Kaur N., Chaudhary J., Jain A., & Kishore, L. 2011. Stigmasterol: a comprehensive review. International Journal of Pharmaceutical Sciences & Research, 2(9), 2259.

Kim Y. S., Li X. F., Kang K. H., Ryu B., & Kim S. K. 2014. Stigmasterol isolated from marine microalgae Navicula incerta induces apoptosis in human hepatoma HepG2 cells. BMB Reports, 47(8), 433.

Li C., Liu Y., Xie Z., Lu Q., & Luo, S. 2015. Stigmasterol protects against Ang II-induced proliferation of the A7r5 aortic smooth muscle cell-line. Food & Function, 6(7), 2266-2272.

O'Callaghan Y. C., Foley D. A., O'Connell N. M., McCarthy F. O., Maguire A. R., & O'brien N. M. 2010. Cytotoxic and apoptotic effects of the oxidized derivatives of stigmasterol in the U937 human monocytic cell line. Journal of Agricultural & Food Chemistry, 58(19), 10793-10798.

Panda S., Jafri M., Kar A., & Meheta B. K. 2009. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from Butea monosperma. Fitoterapia, 80(2), 123-126.

Parrish A. B., Freel C. D., & Kornbluth S. 2013. Cellular mechanisms controlling caspase activation and function. Cold Spring Harbor Perspectives in Biology, 5(6), a008672.

Ramu R., Shirahatti P. S., Nayakavadi S., Vadivelan R., Zameer F., Dhananjaya B. L., & Prasad N. 2016. The effect of a plant extract enriched in stigmasterol and β-sitosterol on glycaemic status and glucose metabolism in alloxan-induced diabetic rats. Food & Function, 7(9), 3999-4011.

Ryan E., McCarthy F. O., Maguire A. R., & O'Brien N. M. 2009. Phytosterol oxidation products: their formation, occurrence, and biological effects. Food Reviews International, 25(2), 157-174.

Shan L. Y., Thing T. C., Ping T. S., Awang K., Hashim N. M., Nafiah M. A., & Ahmad K. 2014. Cytotoxic, antibacterial and antioxidant activity of triterpenoids from Kopsia singapurensis Ridl. Journal of Chemical and Pharmaceutical Research, 6(5), 815-822.

Slee E. A., Adrain C., & Martin S. J. 2001. Executioner caspase-3,-6, and-7 perform distinct, non-redundant roles during the demolition phase of apoptosis. Journal of Biological Chemistry, 276(10), 7320-7326.

Verleyen T., Forcades M., Verhé R., Dewettinck K., Huyghebaert A., & De Greyt W. 2002. Analysis of free and esterified sterols in vegetable oils. Journal of the American Oil Chemists' Society, 79(2), 117-122.

Wang J., Huang M., Yang J., Ma X., Zheng S., Deng S. & Zhao P. 2017. Anti-diabetic activity of stigmasterol from soybean oil by targeting the GLUT4 glucose transporter. Food & Nutrition Research, 61(1), 1364117.

Wong R. S. 2011. Apoptosis in cancer: from pathogenesis to treatment. Journal of Experimental & Clinical Cancer Research, 30(1), 87.

Woyengo T. A., Ramprasath V. R., & Jones P. J. H. 2009. Anticancer effects of phytosterols. European Journal of Clinical Nutrition, 63(7), 813.