Journal for ReAttach Therapy and Developmental Diversities
eISSN: 2589-7799
2022 June; 5 (1): 70-77

Evaluation of Carvacrol Cytotoxicity Depending on Cell Death and Cell Proliferation Markers

Aumaima Tariq Abed[1], Hadeel Abdul Elah Karbel [2]
[1]Department of Pharmacy, Al-Zahrawi University College, Karbala, Iraq
[2]Department of pathology, Hammurabi college of Medicine, Babylon University, Iraq
Email: omaima@g.alzahu.edu.iq

Received: 28-March-2022
Revised: 19-May-2022
Accepted: 30-May-2022

Abstract

Background: Carvacrol is predominant monoterpene presents in the volatile oils of oregano, thyme and some other herbs. It has valuable therapeutic properties that encourage researchers to look into its anticancer activity.

Objectives: The present study was carried out to determine the in vitro cytotoxic and antiproliferative effects of carvacrol on breast cancer cell line.

Materials and Methods: The cytotoxic effect of carvacrol was evaluated by LDH assay. While the antiproliferative effect was assessed as ki-67positive and negative cells. Percentage of growth inhibition was calculated for both tests.

Results: The study clearly showed the cytotoxic and anti-proliferative effect of carvacrol on T47-D cells in dose dependent manner.

Conclusion: These data demonstrated the cytotoxic and antiproliferative of carvacrol on human breast cancer cells, T47-D, and point out that this compound could have a potential therapeutic significance in treating cancer.

Keywords: *carvacrol, cytotoxicity, LDH, proliferation, ki-67, T47-D breast cancer cell line.*

Citation: Abed, T.A., Karbel, A.H. Evaluation of carvacrol cytotoxicity depending on cell death and cell proliferation markers. Journal for ReAttach Therapy and Developmental Diversities, 2022, X

1. Introduction:

Carvacrol (CVL) is a natural member of monoterpene phenol, it is a significant constituent of the essential oils of oregano and thyme (Krause et al., 2021).

Both are of the most vital medicinal plants (Murtada, 2010), they are common in the Maltese Island and Mediterranean Basin, and contain many effective compounds such as thymol and CVL (Raja, 2012).

CVL has been ordinarily used in our life as an ornamental ingredient and nontoxic food additive (Yin, et al., 2012). It has been shown to exhibit antibacterial (Bnyan, et al., 2014) antioxidative, antitussive, antispasmodic and antimicrobial effects (Youdim et al., 1999). These valuable properties have encouraged the researchers to look into its anticancer activity (Arunasree, 2010).

Cancer is one of the major causes of death worldwide. Breakthrough against cancer depends upon both augmented national contribution in cancer research and the submission of existing cancer control knowledge (Siegel, et al., 2016).

The past several decades experienced tremendous developments in cancer research through using cancer cell lines, which count as good medical trial models for cancer study, and have the utmost influence on improving outcome for cancer patients (Holliday and Speirs, 2011).

One of the first stages in finding a better cancer treatment is to test medication candidates using powerful preclinical models. These models should not only identify the best medication options, but also establish if their anticancer impact is strong enough to warrant clinical testing. (Lopez-Lazaro, 2015). Preliminary reports had revealed that CVL exhibited a significant antigenotoxic activity in mammalian cells (Ipek et al., 2003), prevented viability and proliferation of human non-small cell lung cancer in a dose-dependent way (Koparal and Zeytinoglu, 2003), and it is effective against human metastatic breast cancer cells MDA-MB 231 too, hepatocellular carcinoma cell line (Arunasree, 2010), and human cervical cancer cell lines (Cock, 2011). CVL may cause apoptosis by directly activating the mitochondrial and mitogen-activated protein kinase pathways. As a result, it is anticipated that CVL is a strong antitumor drug, although further research is needed. (Yin et al., 2012)

Several cytotoxicity assays are available today, they can be used to detect cell viability through different aspects such as cell integrity, proliferation and metabolic functions (Schroterova et al., 2009).

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in the most types of cells. When cell membrane is damaged, this enzyme is released into the extracellular medium and it can be measured by the spectrophotometer to prove cytotoxicity (Li et al., 2008). LDH is an ideal marker of cell death for in vitro models (Mehdi et al., 2011).

LDH based cytotoxicity assay can improve further quantification without repeating the arrangement and treatment steps, it can be used to identify the total effects, fraction of cell killing and growth inhibition since proliferation assays incapable of differentiating between cell cycle inhibition and cellular death (Smith et al., 2011).

Ki-67 is a cellular proliferation marker, it is a nuclear protein (Licht et al., 1992), which is defined by its reactivity with monoclonal antibody from the Ki-67 clone. It has predictive and prognostic value and a feasible marker for clinical practice that marked all active phases of the cell cycle and degraded as the cell enters the non-proliferative state rapidly. It can be used to estimate treatment response and benefiting from cancer treatment (Fasching et al., 2011).

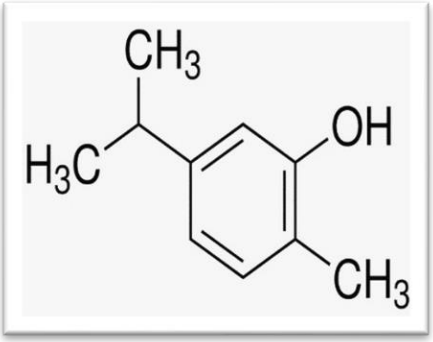

Figure (1)
The structural formula of carvacrol,
(Sigma-Aldrich, 2013)

2. Materials and methods:

Chemicals:

Natural CVL (99%) and RPMI-1640 liquid media were bought from Sigma-Aldrich (USA). Other chemicals and components were bought from local companies and tested previously.

Materials

From natural CVL (99%), serial dilutions were made to obtain the different concentrations which had been used in the assays (800, 400, 200, 100, 50, and 25 µg/ml).

Cell culture and treatment

T47-D cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.5% gentamycin, and 0.2% amphotericin B. Cultures were incubated at 37oC humidified incubator with 5% CO2. The trypan blue dye exclusion technique was used to assess cell viability.

200 µl of 1×106 cells/ml were implanted in flat bottom 96 well microtiter plate, and the plate was incubated at 37oC. After plate incubation, and when confluence reached 80%-90%, the media was removed and washed with 100 µL PBS.

A set of seven replicates was used for the assays, the first replicate was nurtured with RPMI-1640 media to be used as control group, while the other replicates were nurtured with the previously specified concentrations of CVL and incubated for 24 hours at 37oC.

CytotoxicityAssays

Two methods are used to determine the impact of CVL on the viability of T47-D breast cancer cells depending on the cell death and cell proliferation According to the manufacturer´s protocol, all the reagents of the cytotoxicity assays were prepared.

LDH cytotoxicity assay

The cytotoxic effect of CVL toward T47-D breast cancer cells can be determined by measuring the activity of cytoplasmic enzymes released by damaged cells, (LDH). Which acts as a key indicator of cell apoptosis or cell damage (Forkasiewicz et al., 2020).

LDH can be easily quantified by using the formula that mentioned in the kit.

A significant increase in the LDH release in T47-D cells was observed after treatment with CVL in a dose-dependent manner.

PGI (percentage of growth inhibition) was calculated as a fraction of control group (Llobet et al., 2009).

Evaluation of immunohistochemical staining:

Ki-67 can be considered as a prognostic factor for breast cancers. Immunohistochemical evaluation is the most common analysing method of the Ki-67 antigen which can be summerized by using the antihuman Ki-67 monoclonal antibody MIB-1 and reporting the percentage of positively stained malignant cells (Inwald et al., 2013).

The assay was carried out as mentioned by DAKO EnVision FLEX+ protocol detection system.

cells were evaluated under an inverted microscope to realize the expression of Ki-67 proteins.

Alive tumor cells showed a definite brown nuclear staining with Ki-67 antibody were scored as positive (brown nuclear staining means positive stain), while dead cells were scored as negative cells.

The areas with the largest number of cells were used to quantify the positive and negative cells per 600 tumor cells. The Ki67 index was calculated as the percentage of positively and negatively stained cells to the total number of cells in the area (Maryam Kadivar M. and Aram F.,2020).

The percentage of negative cells was calculated by this quantitative method and considered as PGI (Reichert et al., 1998).

Statistical analysis: SPSS version 16 was used to analyze the results. Categorical variables were shown as frequencies and percentages. Continuous variables were shown as mean ± SD with 95% confidence interval. A p-value< 0.05 was considered as significant.

3. Results:

CVL showed dose dependent cytotoxic effect on T47-D breast cancer cells. This effect was evaluated in vitro by two methods.

Lactate dehydrogenase leakage assay:

CVL cytotoxicity on T47-D breast cancer cells was valued in vitro by LDH leakage in culture medium as a response to cell death. This response was calculated as percentage and different concentrations ranging from 25- 800 µg/ml for CVL produced significant difference ($p<0.05$) in the PGI (tables 1), control group was treated with RPMI-1640 medium. PGI was determined for each well as a fraction of the control group. All the results are shown in the table below (table 1).

Evaluation of immunohistochemical staining:

The anti-proliferative effect of CVL was assessed by immunohisto-chemical staining of cells monolayer grown in multi-well plate. Expression of ki-67 (proliferative marker) was seen clearly in wells that treated with RPMI-1640 medium only and did not treated with CVL (figure 2), these wells were used as control group.

Then the expressions were diminished with the increase of the dose of CVL. Faint pictures were seen for high concentrations of CVL treated wells in contrast to the dusky one that treated with low concentrations (figure 3). PGI was calculated quantitatively by counting the number of dead cells which recorded as negative cells per 600 tumor cells.

Table (1): Percentages of growth inhibition produced by different concentrations of carvacrol for the two assays after 24 hours incubation period.

assay / CVL Concentration µg/ml	LDH	Ki-76
Control	1 b,c,d,e,f,g B	1.5 b,c,d,e,f,g A
25	25.50 ±0.30 a,c,d,e,f,g B	24.66±2.08 a,c,d,e,f,g A
50	34.40 ± 0.43 a,b,d,e,f,g B	33.00±3.60 a,b,d,e,f,g A
100	51.00 ± 1.15 a,b,c,e,f,g B	51.66±4.50 a,b,c,e,f,g A
200	67.00 ±0.45 a,b,c,d,f B	64.00±4.35 a,b,c,d,f,g A
400	72.20 ±0.7 a,b,c,d,e, B	64.00±5.29 a,b,c,d,g A
800	70.25±0.68 a,b,c,d, B	72.00 ±3.00 a,b,c,d,e,f A

Different small letters mean significant ($p < 0.05$) difference between different concentrations and control group.
Different capital letters mean non-significant ($p > 0.05$) difference between different methods of assessment.

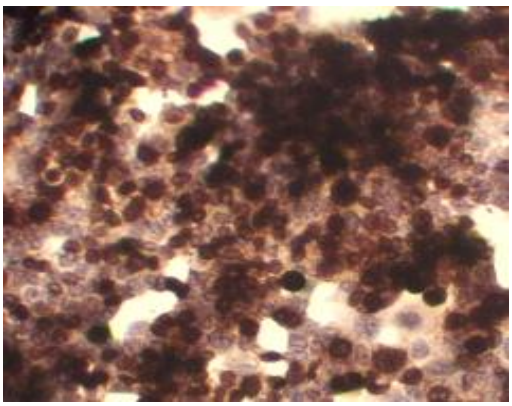

Figure (2): Expression of ki-67 proliferative marker (power 40x 10)

The mean of the two methods was found and used in figure (4) to determine that there is a clear difference in the outcomes for each concentration of CVL.

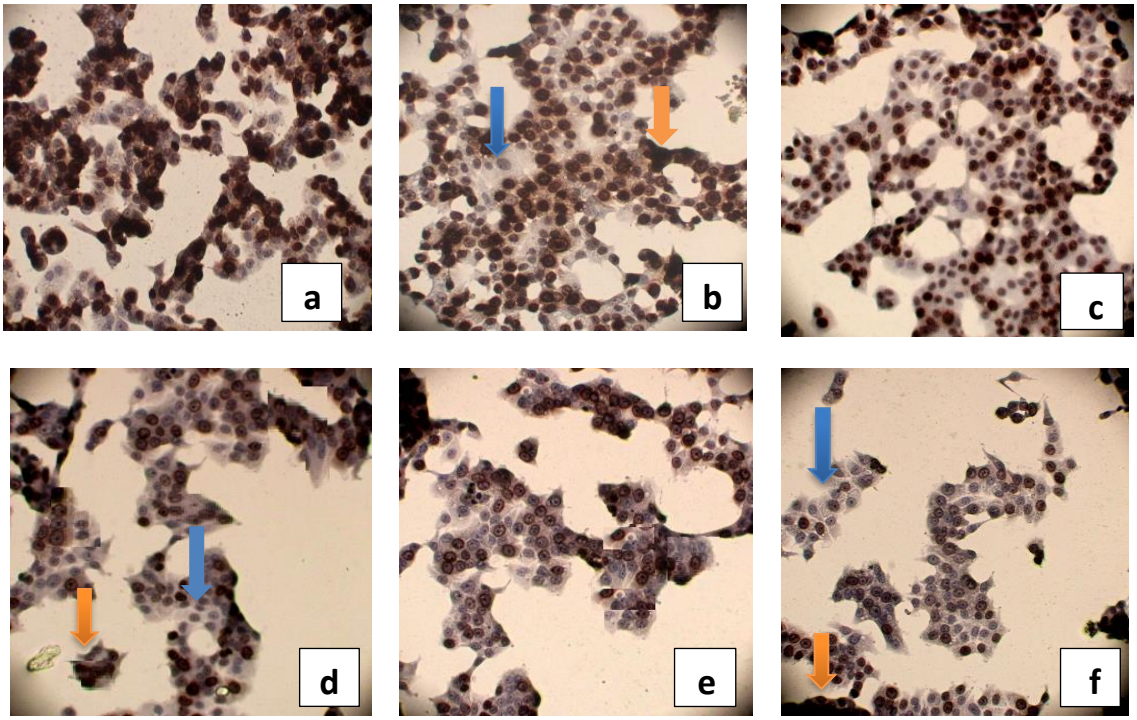

Figure (3): Images showed immunohistochemical staining of T47-D cells stained with Ki-67 proliferative marker after 24 hours exposure to CVL (Power 40x10).
Orange arrows show Ki-67 positive cells, while blue arrows are for Ki-67 negative cells. a: CVL 25 µg/ml, b: CVL 50 µg /ml, c: CVL 100 µg /ml, d: CVL 200 µg /ml, e: CVL 400 µg /ml, f: CVL 800 µg /ml, g:5-FU 25 µg /ml, h: 5-FU 50 µg /ml, i: 5-FU 100 µg /ml, j: 5-FU 200 µg /ml, k: 5-FU 400 µg /ml, l: 5-FU 800 µg /ml.

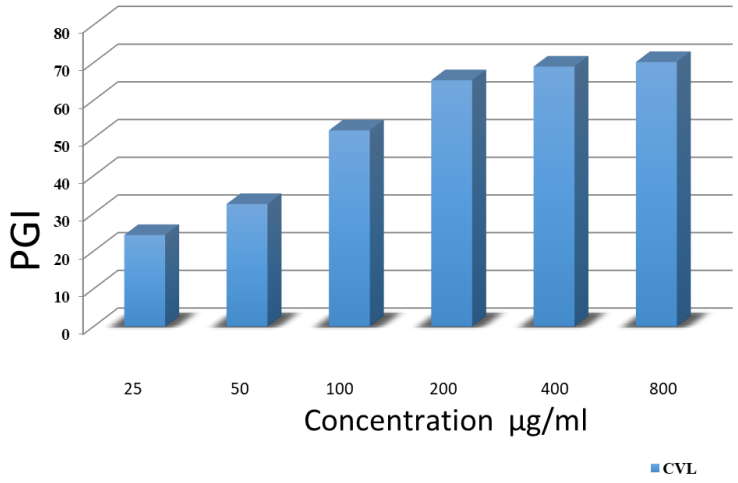

Figure (4): Comparison percentage of growth inhibition produced by different concentrations of carvacrol after 24 hours incubation period.

Data summarized in the table and figures above was showed that PGI of T47-D cells was increased as the dose of CVL increased. These results estimated that those percentages are dose dependent. On the other hand, there was non-significant difference among the results obtained from the two different methods which had been used for CVL cytotoxicity assessment.

4. **Discussion**:

Breast cancer is a complex and heterogeneous disease, which make obstacles both to expecting outcome and to determining an efficient treatment (Holliday and Speirs, 2011).

One important model system for discovering a strong candidate anticancer agent is breast cancer cell lines. Nearly half a century ago or more, cancer cell lines were used to evaluate the clinical value of new investigational anticancer agents and to realize prognostic biomarkers (Sharma et al., 2010). Many ways can be used to explore the cytotoxicity of chemicals on cancer cell lines, such as trypan blue exclusion, C.V, LDH, MTT, and neutral red uptake cytotoxic assays (Freshney, 2010).

The current work shows the anti-proliferative effects of CVL on T47-D breast cancer cells and the possibility of making use of one culture plate to get accurate results for more than one assay.

A previous pilot studies were done to investigate the proper range of concentrations used in this study.

The substantial rise ($p< 0.05$) in PGI of all applied concentrations of CVL for the two different methods of assessment indicated the direct dose dependent cytotoxic effect of CVL. These consequences represents good agreement for the already mentioned cytotoxic effect of CVL on human breast cancer cell line, MDA-MB 231 (Arunasree, 2010) and coincide with the dose dependent anti-proliferative effect of CVL on HepG-2 cells too (Yin et al., 2012) and with the results of Koparal and Zeytinoglu, (2003) who determined that CVL can induce early apoptotic features in a dose-dependent manner, inhibited viability and proliferation of A549 cells Mehdi et al., (2011) indicated that the apoptosis proportion of cells was increased by increasing the dose of CVL in HeLa and SiHa cervical cancer cell lines.

These results can be strengthened with findings of Abid et al., (2014) who determined the impact of CVL on the viability of T47-D cells in dose dependent manner and the results were detected by crystal violet staining assay which coincide with the current study. Also, the non-significant difference among different cytotoxicity tests employed to measure CVL toxicity in vitro matched with Mehdi et al., (2011) findings.

Even though, the LDH leakage assay is based on releasing the enzyme into the culture medium after cell membrane damage, whereas Ki-67 is a proliferation-associated antigen, Ki-67 can provide supplementary and predictive information concerning the response to drug cytotoxicity in vitro, as the two different assays revealed nearly similar results.

So, it might be rational to assume Ki-67 as a predictive marker for in vitro response to newfangled tested cytotoxic agents. Exhaustive study of expression of this proliferation-associated antigen during cell division showed that it is expressed in all active phases of the cell cycle (G0, G1, S, G2). CVL treated cells revealed the rise of G0/G1phase (apoptotic peak) of cell cycle and a reduction of cells at S phase in a concentration dependent manner (Arunasree, 2010)

Moreover, the method of Ki-67 staining and evaluation was routinely used as a part of clinical practice. Ki-67 can be used to detect proliferating tumor cells in tumor tissue as well as in tumor cell lines (Reichert et al., 1998).

This work obviously directs that CVL produced significant ($p< 0.05$) decrement in the viability of T47-D cells and it had a strong anti-tumor molecule against breast cancer cells which agree with the results of Jaafari et al., (2012) who determined that monotrpenes like CVL had antitumor activity when studied separately.

The aim of cancer treatment is to terminate or at least control the progress of neoplastic cells without suggestively touching the viability and function of host cells (Markman 2006). Rapid advances in cancer medicine have produced a numerous newly agents. New therapeutic modalities have been advanced with the aim of encouraging apoptosis since most current therapies lead to cell death mediated by apoptosis as well as necrosis (Tannock, et al, 2005). Many chemotherapeutic agents for cancer have diverse considerable short- and long-term side effects. Thus, recently many researches have been attentive on contents isolated from herbs and plants which have been considered for being nontoxic, prevention and treatment of certain types of cancer. Recently, the latest researches presented that the anti-proliferative effect of CVL on breast cancer cells was built on the activation of the classical apoptosis response (Arunasree, 2010).

Despite substantial advances in our knowledge of the molecular basis of cancer and the backing of a number of molecularly targeted medicines, the clinical drug development process remains slow, expensive, and inefficient (Bielack, 2010). Because carcinogenesis is well known as a complicated and multistep process in which oxidative stress and inflammation play important roles and various molecular and

cellular changes occur. CVL has antimutagenic (Ipek et al., 2003), anti-inflammatory (Landa et al., 2009), and antioxidant properties in addition to its cytotoxic impact, making it a unique anticancer therapeutic (Kim et al., 2013).

On the other hand, current research indicates that cancer is a chronic disease, similar to hypertension or rheumatoid arthritis, and patients should be on lifelong maintenance treatment (Marsh and Samuel, 2007). Assessing effectiveness and adverse effects is thus a major challenge in the treatment of breast cancer (Cai et al., 2009). For a drug license to be granted, a manufacturer usually requires to prove the safety and efficacy of a drug for a given indication (Hall et al., 2010). CVL is approved by the FDA for food use and was included by the Council of Europe, in the list of chemical flavorings, category B, which may be added to foodstuffs (De Vincenzit et al., 2004).

All these findings suggested that CVL can give a spark of hope in cancer therapy.

5. Conclusions:

1. CVL has an in vitro cytotoxic effect against T47-D human metastatic breast cancer cells.
2. Two methods (LDH, and proliferative marker Ki-67) for CVL cytotoxicity assessment gave statistically similar results.
3. Prospective and extensive studies are required on laboratory animals to prove the in vivo efficacy of CVL.

6. Recommendation:

1. It seems to be necessary to conduct further studies involving the pharmacokinetic of CVL, since there are limited references available about this matter.
2. Future investigations should consider whether this effect is due to CVL only, if not the crude extracts of oregano or thyme may possess the same.

References:

[1]. Abid, AT.; Al-Kafaji B.; Karbel H. and Naji H. (2014). Damper Effect of Carvacrol on T47-D, A Human Breast Cancer Cell Line. Medical Journal of Babylon-Vol. 11- No. 4 -2014.

[2]. Arunasree, KM. (2010). Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. Phytomedicine. 17(8): 581-588.

[3]. Bielack, SS. (2010). Osteosarcoma: Time to move on? EJC. 46(11): 1942-1945.

[4]. Bnyan, IA.; Abid AT. and Obied HN. (2014). Antibacterial Activity of Carvacrol against Different Types of Bacteria. Journal of Natural Sciences Research. 4(9): 13-16.

[5]. Cai, H.; Marczylo, TH.; Teller, N.; Brown, K.; Steward, WP.; Marko, D. and Gescher, AJ. (2009). Anthocyanin-rich red grape extract impedes adenoma development in the Apc min mouse:Pharmacodynamic changes and anthocyanin levels in the murine biophase. EJC. 46(4): 811-817.

[6]. Cock, I.E. (2011). Problems of Reproducibility and Efficacy of Bioassays Using Crude Extracts, with reference to Aloe vera. Pharmacognosy Communications. 1(1):52-62.

[7]. De Vincenzit, M.; Stammati, A.; De Vincenzi, A. and, Silano, M. (2004). Constituents of aromatic plants: carvacrol. Fitoterapia 75: 801– 804.

[8]. Fasching, PA.; Heusinger, K.; Haeberle, L.; Niklos, M.; Hein, A.; Bayer, CM.; Rauh, C.; Schulz-Wendtland, R.; Bani, MR.; Schrauder, M.; Kahmann, L.; Lux, MP.; Strehl, JD.; Hartmann, A.; Dimmler, A.; Beckmann, MW and Wachter, DL. (2011). Ki67, chemotherapy response, and prognosis in breast cancer patients receiving neoadjuvant treatment. BMC Cancer; 11:486-499.

[9]. Forkasiewicz, A.; Dorociak, M.; Stach, K.; Szelachowski, P.; Tabola, R.; Augoff, K. The usefulness of lactate dehydrogenase measurements in current oncological practice. Cell. Mol. Biol. Lett. 2020, 25, 35. [Google Scholar] [CrossRef]

[10]. Freshney, RI. (2010). Culture of Animal Cells. a Manual of Basic Technique and Specialized Applications., 6th ed. John Wiley & Sons. Inc. Hoboken. New Jersey.

[11]. Hall, PS.; McCabe, C.; Brown, J.M. and Cameron, DA. (2010). Health economics in drug development: Efficient research to inform health care funding decisions. EJC. 46(15): 2674-2680.

[12]. Holliday, DL. and Speirs, V. (2011). Choosing the right cell line for breast cancer research. Breast Cancer Research. 13:215.

[13]. Inwald, E.C.; Klinkhammer-Schalke, M.; Hofsta¨dter, F.; Zeman, F.; Koller, K.; Gerstenhauer, M. et al. (2013). Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. Breast Cancer Res Treat 139:539-552 [DOI:10.1007/s10549-013-2560-8] [PMID] [PMCID]

[14]. Ipek, E.; Tuylu, BA. and Zeytinoglu, H. (2003). Effects of carvacrol on sister chromatid exchanges in human

lymphocyte cultures. Cytotechnology. 43:145–148.

[15]. Jaafari, A.; Tilaoui, M.; Ait Mouse, H.; M'bark, L.A.; Aboufatima, R.; Chait, A.; Lepoivre, M. and Zyad, A. (2012). Comparative study of the antitumor effect of natural monoterpenes: relationship to cell cycle analysis. Brazilian Journal of Pharmacognosy. 22:534-540.

[16]. Kadivar M. and, Aram f. (2020). Assessment of Ki67 in Breast Cancer: A Comparison Between the Eye-10 Method, Stepwise Counting Strategy, and International System of Ki67 Evaluation. Iran J Pathol.; 15(1): 13-18

[17]. Kim, E.; Choi, Y.; Jang, J. and Park, T. (2013). Carvacrol protects against hepatic steatosis in mice fed a high-fat diet by enhancing S1RT1-AMPK signaling. Evidence- Based complementary and Alternative Medicine. 13: 10.

[18]. Koparal, AT. and Zeytinoglu, M. (2003). Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549. Cytotechnology 43: 149–154.

[19]. André Sanches Fonseca Sobrinho. (2020). An Embedded Systems Remote Course. Journal of Online Engineering Education, 11(2), 01–07. Retrieved from http://onlineengineeringeducation.com/index.php/joee/article/view/39

[20]. Krausea, ST. Liaob, P. Crocollc, C. Boachon, B. Forster, C. Leideckera, F. Wiesea, N. Zhaoe, D. Woode, JC. Buelle, R. Gershenzonc, J. Dudareva, N. and Degenhard J. (2021). The biosynthesis of thymol, carvacrol, and thymohydroquinone in Lamiaceae proceeds via cytochrome P450s and a short-chain dehydrogenase. PNAS Vol. 118 No. 52 e2110092118

[21]. Jamiatul Hasanah Siregar. (2022). Tree Stratification in the Permanent Plot of HPPB Andalas University Padang. Revista Electronica De Veterinaria, 25 - 32. Retrieved from https://www.veterinaria.org/index.php/REDVET/article/view/136

[22]. Landa, P.; Kokoska, L.; Pribylova, M.; Vanek, T.; and Marsik, P. (2009). In vitro Anti-inflammatory Activity of Carvacrol: Inhibitory Effect on COX-2 Catalyzed Prostaglandin E2 Biosynthesis. Arch Pharm Res. 32(1): 75-78.

[23]. Li, Q.; Kobayashi, M. and Kawada, T. (2008). Chlopyrifos induces apoptosis in human T cells. Toxicology. 255(1-2):53-57.

[24]. Licht, T.; Bross, KJ.; Fiebig, HH.; Schotta, K.; Berger, DP.; Dreher, C.; Lohr, GW. and Herrman, F.(1992). Expression of the proliferation-associated Ki-67 antigen of transferrin receptors and of DNA polymerase alpha in human tumor lines: implications for in vitro chemoresistance. J Cancer Res. Clin. Oncol. 118(2):116-122.

[25]. Llobet D.; Eritja, N.; Yeramian, A.; Pallares, J.; Sorolla, A.; Domingo, M.; Santacana, M.; Gonzalez-Tallada, F.J.; Matias-Guiu, X. and Dolcet, X.(2009). The multikinase inhibitor Sorafenib induce apoptosis and sensitizes endometrial cancer cells to TRAIL by different mechanisms. EJC. 46(4): 836-850.

[26]. Lopez-Lazaro, M. (2015). Two preclinical tests to evaluate anticancer activity and to help validate drug candidates for clinical trials. Oncoscience. 2(2): 91–98

[27]. Markman, M. (2006). The Elsevier guide to oncology drugs and regimens. 1st edition. Elsevier. Huntington. USA. Part 1 and 2.

[28]. Marsh, RDW. and Samuel, J. (2007). Essentials of Clinical Oncology. Mc Graw Hill. Section 1,2,3.

[29]. Mehdi, SJ.; Ahmad, A.; Irshad, M.; Manzoor, N. and Rizvi, MMA. (2011). Cytotoxic effect of Carvacrol on human cervical cancer cells. Biology and Medicine, 3 (2):307-312.

[30]. Murtada, JH. (2010). Study effect of aqueous Origanum Vulgare L. extract and Rhodium(III) complex on cancer cell line. Iraqi Journal of Biotechnology. 9(4): 729-740.

[31]. Raja RR. (2012). Medicinally Potential Plants of Labiatae (Lamiaceae) Family: An Overview. Research Journal of Medicinal Plants, 6(3): 203-213.

[32]. Reichert, TE.; Watkins, S.; Stanson, J.; Johnson, JT.and Whiteside, TL. (1998). Endogenous IL-2 in Cancer Cells: A Marker of Cellular Proliferation. Journal of Histochemistry & Cytochemistry. 46(5): 603–611.

[33]. Schroterova, L.; Kralova, V.; Voracova, A.; Haskova, P.; Rudolf, E. and Cervinka, M. (2009). Antiproliferative effects of selenium compounds in colon cancer cells: Comparison of different cytotoxicity assays. Toxicology in Vitro. 23(7): 1406-1411

[34]. Sharma, SV.; Haber, DA. and Settleman, J. (2010). Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nat Rev Cancer. 10(4):241-253.

[35]. Siegel, RL. ; Miller KD. and Jemal A. (2016). Cancer statistics, 2016. CA Cancer J Clin. 66(1):7-30

[36]. Sigma-Aldrich. Chemie GmbH. Riedstrasse 2. D-89555 STEINHEIM. (2013). Safety data sheet.

[37]. Smith, SM.; Wunder, MB.; ,Norris DA.; and Shellman YG.; (2011). A Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. http://journals.plos.org

[38]. Tannock, IF.; Hill, RP.; Bristow, RG. and Harrington, L. (2005). The Basic Science of Oncology. (4th edition). McGraw-Hill. Chapter 3,10,16.

[39]. Yin, Q.; Yan, F.; Zu, X.; Wu, Y.; Wu, X.; Liao, M.; Deng, S.; Yin, L. and Zhuang, Y. (2012). Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology. 64(1): 43-51.

[40]. Youdim, K.A., Damien, H.J. and Deans, S.G. (1999). The antioxidant effectiveness of thyme oil, α- tocopherol and ascorbyl palmitate on evening primrose oil oxidation. J. Essential oil Res. 11(5): 643-648.