DOI Number: 10.37506/v14/i1/2020/ijfmt/193002

Broad-Spectrum Cytotoxic Effect of *Calendula officinalis L* Against Breast Cancer Cells

Khulood M. Alsaraf[1], Maeda H. Mohamed[1], Ahmed Majeed Al-Shammari[2], Ibrahim S. Abbas[3]

[1]Pharmacy department, Al-Esraa University College: Baghdad, Iraq, [2]Experimental Therapy Department, Iraqi Center for Cancer and Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq, [3]Department of Pharmacognosy and Medicinal Plants, College of pharmacy, Mustansiriyah University, Baghdad, Iraq

Abstract

Background: *Calendula officinalis L* used in Iraqi folklore medicine for several medical applications. This research evaluated the leaves extract as an anti-breast cancer agent in in-vitro cancer cell line systems and studies its active compounds. Crystal violet viability assay was used to determine the cytotoxicity of the leave methanolic extract of *Calendula officinalis L* against diverse breast cancer cell lines. Human breast cancer MCF7, AMJ13, MDAMB, and CAL51 cells were treated with different concentrations of extract for 72 hours. Morphological study for the exposed cell was done by examination under a phase-contrast inverted microscope. High-performance liquid chromatography (HPLC) analysis was performed to measure the concentrations of each component of phenols and flavonoids in the *Calendula officinalis L* extract. **Results:** It was found that methanolic extract of *Calendula officinalis L* inhibits the proliferation of all breast cancer cells significantly at the meantime; it does not affect normal embryonic cells. Additionally, it induced the cytopathic morphological changes in cancer cells. Furthermore, HPLC study revealed that *Calendula officinalis L* extract contained an important component of flavonoids. **Conclusions:** *Calendula officinalis L* leaves extract inhibited the proliferation of breast cancer cells especially MDAMB cells with no effect on normal cells. This work showed that *Calendula officinalis L* is a possible natural source as broad-spectrum anti-breast cancer drug.

Keywords: *Cytotoxicity; HPLC analysis; Flavonoids; Iraq; Clonogenic assay*

Introduction

Cancer disease is a highly complex condition that hard to treat (1). Its incidence is increased globally and especially in Iraq due to several factors mainly related to environmental pollution for several years of war conflicts (2). Breast malignant tumours are ranking second causing of mortality in Iraqi females. Conventional cancer treatments such as chemotherapy, radiation, targeted therapy; immunotherapy, etc, have their unwanted side effects. Therefore, herbal medicine has shown as a useful alternative for the present therapies (3, 4). Herbal medicine widely used for the treatment for several types of diseases such as viral infections and cancer as it has fewer side effects that may be caused by conventional cancer therapeutics (5-7). Marigold (*Calendula officinalis L.*) belong to Asteracea family and was considered among the most important medicinal and garden plants (8). Several species of this plant are widely distributed in different Mediterranean countries. Marigold is an aromatic annual, seldom biennial. It grows between 30 and 50 cm height and has about 20 cm long tap root and numerous thin secondary roots (9). The stem is erect, angular, down, and branched from the base up or higher. The alternate leaves are almost spatulate at the base, oblong to lanceolate above and are all tomentosae, Several phytochemical studies have been established to investigate the presence of numerous classes of chemical compounds. The main

Corresponding author:
Ahmed Majeed Al-Shammari,
Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic research, Mustansiriyah University. Email: ahmed.alshammari@iccmgr.org, tell: 009647809143825.

compounds are terpenoids, flavonoids, coumarines, quinones, volatile oil, carotenoids, and amino acids (10, 11). Pharmacological studies have confirmed that C.officinalis shows a wide range of biological effects such as anti-inflammatory, antioxidant, hepatoprotective, and immunostimulant activities (12, 13). Cytotoxic effect of C.officinalis on tumour cell lines in vitro and its anticancer efficacy in an in vivo briefly outlined 20 years ago (14). According to the most active compounds that have high bioactivity this research was conducted to evaluate the leaves extract as an anti-tumor agent in in vitro cancer cell line systems.

Materials and Method

Collection of plant samples:

Flowers of the marigold plant were collected from medical plants garden at the college of pharmacy. The collected flowers were authenticated and did the formal identification of the plant material by the National Herbarium Centre in Abu – Graib countryside/Iraq and a specimen of this material has been deposited in its data base. Furthermore, the flowers were washed under running tap water to remove the surface pollutants, dried for two weeks at room temperature in the shade, then after, grinded to fine powder, weighed, and stored for future studies at room temperature.

Plant extraction:

100 grams of flower powder was extracted by using soxhlet apparatus in the presence of ethanol 90 (500 ml) till exhaustion. The extract was concentrated by using rotary evaporated, then mixed with 50 mL of distilled water and extracted with 30 mLX 3 of ethyl acetate. The upper layer which was ethyl acetate layer was separated by a reparatory funnel, then dried by using anhydrous sodium sulphate, and labelled as Ethyl acetate extract (15).

Method of Analysis

Phenols & Flavonoids in *Calendula* Extract:

Analysis of phenols & flavonoids in *Calendula officinalis* was performed by HPLC for the detection of flavonoid. A 3 micrometre particle size Column (50*4.6 mm 1.D) Shimpack C-18 with a mobile phase of 0.1% phosphoric acid : acetonitrile (52:24, V\V), and a detection UV set at 285nm. The flow rate is 1.5 ml / mainland at a temperature of 25□. The concentration for each compound were quantitatively determined by comparison the peak area of standard with that of the sample (16)

Maintenance of cell cultures

The human breast cancer cell lines AMJ13 (17), MCF7, MDAMB, CAL51 and the mouse embryo fibroblast (MEF). The AMJ13 cell line was cultured in an RPMI-1640 medium (USbiological, USA) with 10% fetal bovine serum (FBS) (Capricorn- Scientific, Germany), 100 units/mL penicillin, and 100 µg/mL streptomycin. A human breast cancer cell lines MCF7, MDAB, CAL51, were cultured in MEM medium (USbiological, USA) supplemented by 10% fetal bovine serum (FBS) (Capricorn- Scientific, Germany), and 100 µg/mL streptomycin, 100 units/mL penicillin. The cells were incubated at 37 °C in a humidified environment and 5% CO2 (17) for 72 hours.

Cytotoxicity Assays

Crystal violet cell viability assay was employed to measure the cytotoxic effect of plant extract. Human Breast cancer cell lines (MDAMB, AMJ13, MCF7, and CAL51), as well as normal mouse embryonic cells (MEF), were seeded at 7000 cells/well in 96-well plates (Santa Cruz Biotechnology, USA), after 24hr or until confluent monolayer is achieve. Cells were treated with (extract) at 2 fold dilutions from 4000, 2000, 1000, 500, 250, 125, 62.5, 31,25, µg to 15µg of culture media. The assay was done in triplicate and the cell viability was determined after 72h of exposure by staining with 50 µl of Crystal violet (Sigma Aldrich, USA) and incubated at 37°C for 2h. The stain was aspirated, and PBS used to wash the wells. The microplate reader (Biochrom, UK) was used to measure the absorbency at 492 nm; Results were shown percentage proliferation with respect to control cells (18, 19).

Morphology analysis

The treated and untreated cells were photographed at a magnification of 200x at four haphazardly selected cultured fields using an inverted light microscope (Leica-microsystems, Germany) and a digital colour camera (Leica-microsystems, Germany) (20).

Statistical Analysis

The data of the current study are presented as means ± standard error of the mean. One-way analysis of variance was used for data comparison between treatment groups. Data were considered statistically

significant at $P < 0.05$. A GraphPad Prism 6 software was used for the analysis (GraphPad Software, Inc. San Diego, California).

Results

Chemical structure analysis *Calendula officinalis L*:

The HPLC results were shown the different bioactive compound such us flavonoid glycosides were included vitexin, Rutin, qnercetin -3- glycoside, Luleolin -7- glycoside, quercetin -3- glucoside quercitrin, myricetin, Luteolin, and Apigenin. Table 1 and Figure1.

Table 1: HPLC results for *Calendula officinalis L* flower extract

	Standard			Sample		
Subjects	**Retention time (min)**	**Area**	**Conentration mg\ ml**	**Retetion time**	**Area**	**Consentrctin mg\ml**
vitexin	2.005	447823	11.30	1.975	292548	5.35
Rutin	2.910	482715	12.21	2.872	183891	3.38
Qercetin-3 galactoside	4.510	496577	12.60	4.45	327994	6.17
Luleolin-7-glucoside	5.515	364478	9.29	5.528	823263	15.01
Quercetin -3-glucoside	6.683	290249	7.38	6.677	334658	6.13
Quercitrin	8.018	386323	9.38	8.812	1858788	33.03
Myricetin	9.587	392546	10.65	9.500	1288788	23.41
Luteolin	10.793	421348	10.76	10.735	188879	3.46
Apigenin	11.745	278269	7.08	11.723	48132	1.89
Kaempferol	12.695	368117	9.35	12.648	118499	2.17

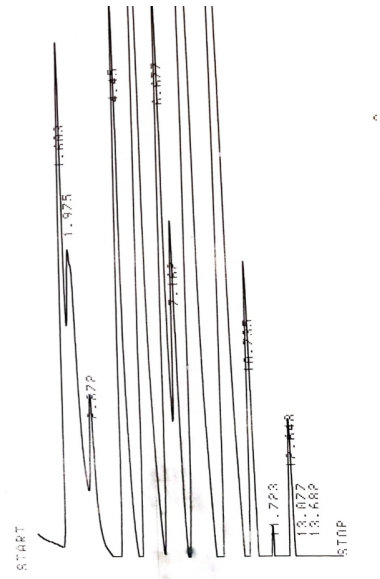

Figure 1: HPLC chromatogram analysis of Calendula officinalis L.

Cytotoxicity assay

The current study investigated the selective cytotoxic effect of the Calendula officinalis L. extract in breast cancer cells. In this study, four human breast cancer cell lines were used, CAL51, MCF7, AMJ13, and MDAMB, and the normal mice embryonic cells, MEF. All cell lines were exposed to Calendula officinalis L. extract at 2-fold concentrations started from 0.0 µg/mL to 4mg for 72 h, and cytotoxicity was determined using crystal violet assays. As shown in Figure-2a, the Calendula officinalis L. extract had no cytotoxic effect on the normal cells as the IC50 was very high dose (4440mg/ml) compared to the IC50 on cancer cells which were 2088µg, 1737µg, 3081µg and 4.732µg for the AMJ13, MCF7, CAL51, and MDAMB, respectively. These results indicate that Calendula officinalis L. extract is very effective against MDAMB cells as revealed by Figure-3.

Cytopathological observation showed that Calendula officinalis L extract-treated cells had lower cell count due to detachment in compare to the control (not treated cells). Furthermore, there were condensed nuclei which refer to early apoptosis in the treated cell compared to untreated cells and this photo is shown in the highest concentration used of exposure. Untreated cancer cells continue to proliferate to form monolayers. Early apoptotic cells that have condensed nuclei and stained darker along with normal lightly stained cells (Figure-4).

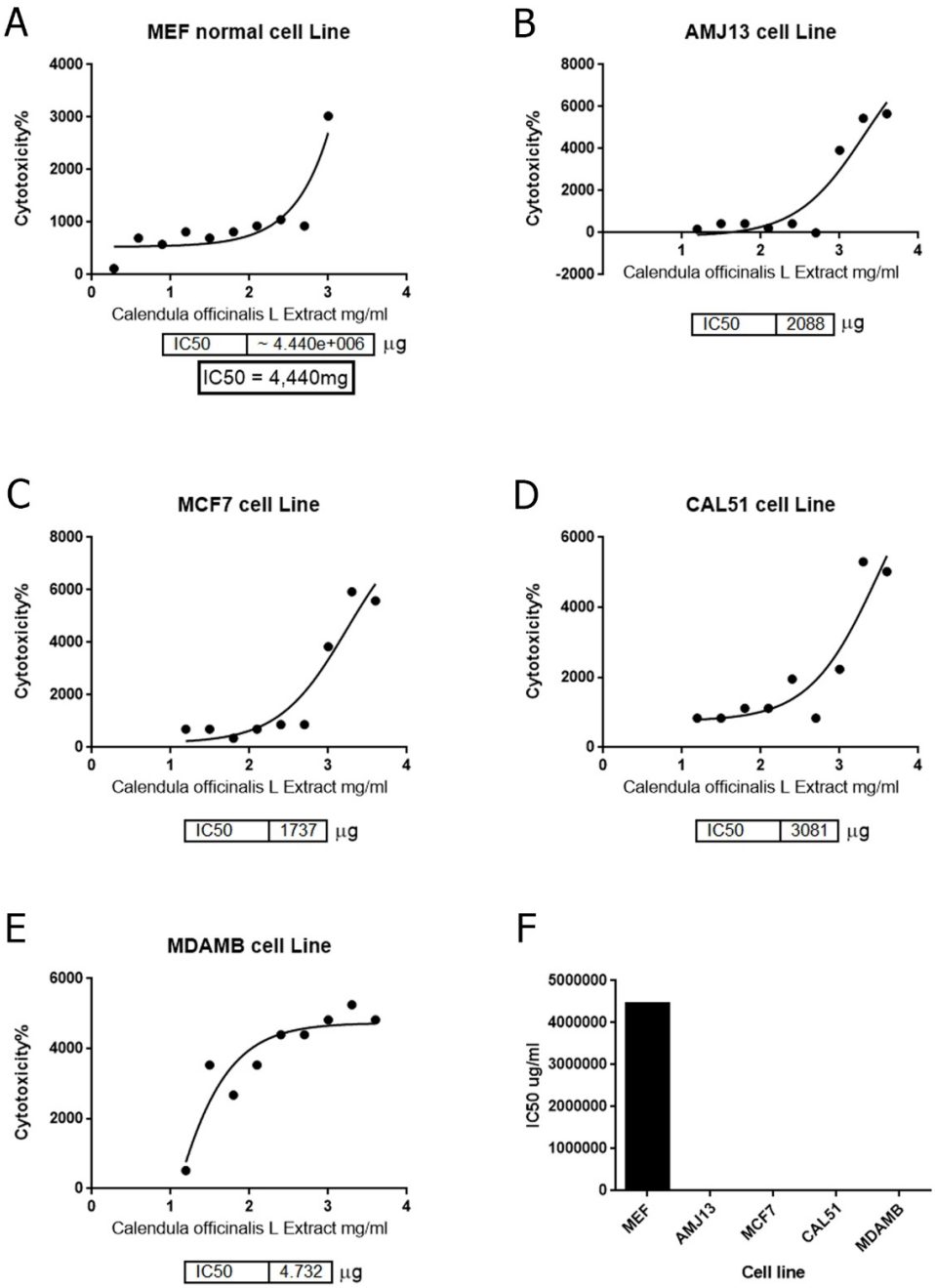

Figure-2, The Calendula officinalis L extract cytotoxicity assay. A) showed no cytotoxicity against normal mouse embryonic cells as the IC50 was very high dose 4440mg, while against breast cancer cell lines were very low IC50 values. B) AMJ13 the IC50 value is 2088 µg/ml. C) MCF7 IC50 is 1737 µg/ml. D) IC50 value in CAL51 cells was 3081 µg. E) IC50 in MDAMB cells was 4.732 mg/ml. F) the comparative study for IC50 values showed that cancer cells are very sensitive to the Calendula officinalis L extract in comparison to the normal embryonic cells.

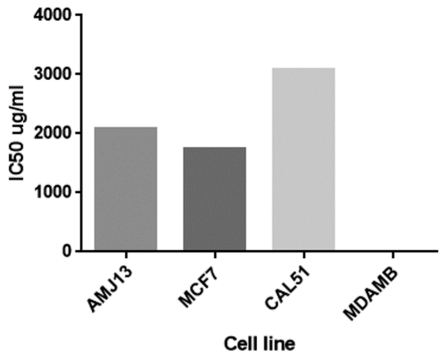

Figure-3, Comparison between cancer cell lines according to their sensitivity to the Calendula officinalis L. extract. The figure indicates that Calendula officinalis L. extract is very effective against MDAMB cells, and this cell line is very sensitive to the extract more than other cells types.

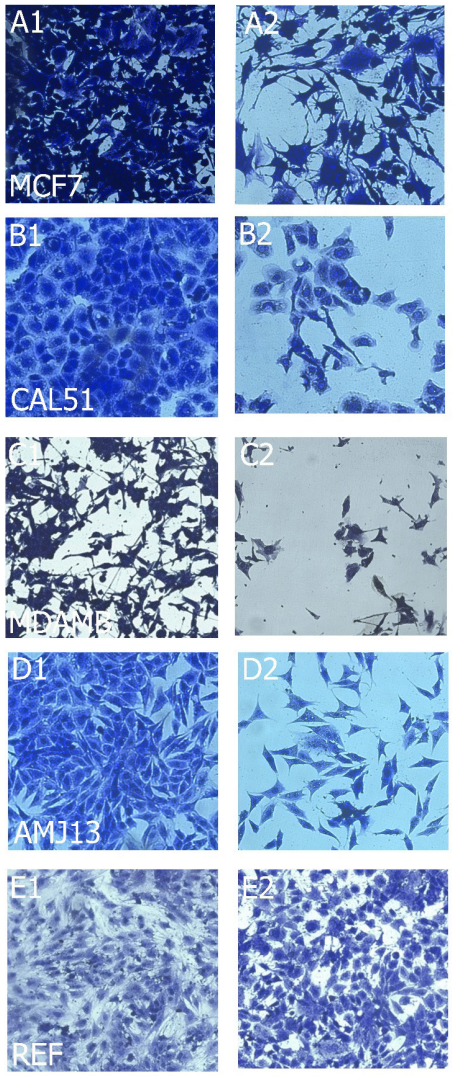

Figure-4, Cytomorphology of treated and control cells. A) MCF-7, A1 control, A2 treated cells showing the extract induces cell death. B) CAL51, B1 control, B2 treated, showing the extract induce cell shrinkage. C) MDA MB-468,

Cont ... Fig 4

C1 control, C2 treated, the extract induces cell and nuclear condensation. D) AMJ13, D1 control, D2 treated, the extract reduced cancer cells number due to detachment. E) MEF, E1 control, E2 treated, E3 image analysis showing a high dose of extract was cytotoxic on normal cells. 400xg (Crystal violet stain)

Discussion

HPLC analysis for Calendula officinalis L. extract revealed the presence of active compounds mainly flavonoids. Active compounds have been recognized and isolated to be used in cancer therapy (21). Flavonoid compounds were founds to be the major constituent of the extract as revealed by HPLC analysis. There is over 4000 type of flavonoids; several of them are accountable for the beautiful colors of fruits, flowers, and leaves (22). The scavenging of oxygen-derived free radicals is a significant effect of flavonoids that also showed anti-carcinogenic properties (23). The antioxidative effect is the top-defined feature of nearly every group of flavonoids. The flavones and chaechins are flavonoids that protect the body from reactive oxygen species (ROS). Cells organelles and components can be damaged by ROS, and free radicals, that are induced by exogenous damage or produced during the metabolism of oxygen (24, 25). Throughout injury, production elevation of reactive oxygen species results in consumption and exhaustion of the endogenous scavenging compounds. Flavonoids may have an additive effect on the endogenous scavenging compounds (26). Our experiments outcomes of cytotoxicity assay revealed that Calendula officinalis L. extract have antiproliferative and cytotoxicity against breast carcinoma cell lines, especially the MDAMB breast cancer cells. Other researchers found that Calendula officinalis extracts also had cytotoxicity on human melanoma and epidermoid carcinoma cells (27). The cytotoxic effect of Calendula officinalis L. extract was explained by the presence of the major flavonoids, which are flavone and luteolin-7-O-β-glucoside (28). Other research found that that luteolin-7-O-β-glucoside is promising anti-cancer molecule, that possesses anti-breast adenocarcinoma (29). C officinalis described as important agent for developing novel cancer therapeutics, moreover, it used to reduce the side effects of radiotherapy (8).

Conclusions

In Conclusion, we reported for the first time that Iraqi Calendula officinalis L. extract is selective broad spectrum anti-breast cancer agent different type breast

cancer cells such as estrogen progesterone positive or triple negative breast cancer and has no toxic effect on normal cells which make it very promising candidate as cancer therapy for clinical application.

Abbreviations

(HPLC) High-performance liquid chromatography,

(IC50) 50% Inhibition of cell lines growth,

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

References

1. O'Connor CM, Adams JU, Fairman J. Essentials of cell biology. Cambridge, MA: NPG Education. 2010;1.
2. Al-Shammari AM. Environmental pollutions associated to conflicts in Iraq and related health problems. Reviews on Environmental Health2016. p. 245.
3. Alsabah AS, Abd AH, Al-Shammari AM. Cytotoxicity of Xanthium Strumarium against Breast Cancer Cell Lines. Journal of Global Pharma Technology. 2018;10(3):767-76.
4. Al-Shamery AM, Yaseen NY. Growth inhibitory effect of fresh garlic extracts on transplanted mammary adenocarcinoma in mice. Iraqi Journal of Cancer. 2009;2(1):40-5.
5. Salih RH, Odisho SM, Al-Shammari AM, Ibrahim OMS. Antiviral effects of olea europaea leaves extract and interferon-beta on gene expression of newcastle disease virus. Adv Anim Vet Sci. 2017;5(11):436-45.
6. Jassim AN, Al-Shammari AM, Abd Al-Hameed H. Antiviral activity of Arctigenin against Newcastle Disease virus in vitro. Research Journal of Chemistry and Environment. 2019;23(S1):68-76.
7. Al-Hilli Z, Hamza A, Al-Jumaily E, Yaseen N, editors. The Antiangiogenic effect of polyphenolic fraction of Cyperus rotundus L. on Human Glioblastoma cell line. First Scientific Conference on Nanotechnology, Advanced Material and Their applications, At University of Technology, Baghdad, Iraq; 2009.
8. Cruceriu D, Balacescu O, Rakosy E. Calendula officinalis: Potential Roles in Cancer Treatment and Palliative Care. Integrative cancer therapies. 2018;17(4):1068-78.
9. Wichtl M. Herbal drugs and phytopharmaceuticals: a handbook for practice on a scientific basis: Medpharm GmbH Scientific Publishers; 2004.
10. Wilkomirski B, Kasprzyk Z. Free and ester-bound triterpene alcohols and sterols in cellular subfractions of Calendula officinalis flowers. Phytochemistry. 1979;18(2):253-5.
11. Okoh O, Sadimenko A, Afolayan A. The effects of age on the yield and composition of the essential oils of Calendula officinalis. J Appl Sci. 2007;7(23):3806-10.
12. Della Loggia R, Becker H, Issac O, Tubaro A. Topical anti-inflammatory activity of Calendula officinalis extracts. Planta Médica. 1990;56(06):658-.
13. Ukiya M, Akihisa T, Yasukawa K, Tokuda H, Suzuki T, Kimura Y. Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (Calendula officinalis) flowers. Journal of natural products. 2006;69(12):1692-6.
14. Boucaud-Maitre Y, Algernon O, Raynaud J. Cytotoxic and antitumoral activity of Calendula officinalis extracts. Die Pharmazie. 1988;43(3):220-1.
15. Alsaffar DF, Abbas IS, Dawood AH. Investigation of the Main Alkaloid of London Rocket (Sisymbrium irio L) as a Wild Medicinal Plant Grown in Iraq. 2016.
16. Kadhum HH, Abd AH, Al-Shammari AM. HPLC analysis and chemical composition identification of isolated flavonoid fraction of Althaea officinalis from Iraq. AIP Conference Proceedings. 2019;2123(1):020045.
17. Al-Shammari AM, Alshami MA, Umran MA, Almukhtar AA, Yaseen NY, Raad K, et al. Establishment and characterization of a receptor-negative, hormone-nonresponsive breast cancer cell line from an Iraqi patient. Breast Cancer : Targets and Therapy. 2015;7:223-30.
18. Al-Khafaji AS, Ahmed M, Firas S. Photodynamic action of low power He-Ne laser on photosensitized human Hep-2 and AMN3 cell lines with

hematoporphyrin derivative in vitro. Iraqi J of Canc and Med Gen. 2010;3(1):54-60.

19. Al-Shammari AM, Salman MI, Saihood YD, Yaseen NY, Raed K, Shaker HK, et al. In vitro synergistic enhancement of Newcastle Disease Virus to 5-fluorouracil cytotoxicity against tumor cells. Biomedicines. 2016;4(1):3.
20. Al-Shammari AM, Syhood Y, Al-Khafaji AS. Use of low-power He-Ne laser therapy to accelerate regeneration processes of injured sciatic nerve in rabbit. The Egyptian Journal of Neurology, Psychiatry and Neurosurgery. 2019;55(1):1.
21. abdul Jalill RD, Hussein MF, Al-Shammari AM. GC/MS ANALYSIS OF RHEUM RIBES RHIZOMES. Mintage Journal of Pharmaceutical and Medical Sciences. 2015:29-34.
22. de Groot H, Rauen U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. Fundamental & Clinical Pharmacology. 1998;12(3):249-55.
23. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. Journal of nutritional science. 2016;5:e47-e.
24. Jaafar NS, Hamad MN, Abbas IS, Jaafar IS. Qualitative phytochemical comparison between flavonoids and phenolic acids contents of leaves and fruits of Melia azedarach (Family: malaceae) cultivated in Iraq by HPLC and HPTLC. International J of pharmacy and pharmaceutical science. 2016;8(10).
25. Jung W, Chung I, Kim S, Kim M, Ahmad A, Praveen N. In vitro antioxidant activity, total phenolics and flavonoids from celery (Apium graveolens) leaves. Journal of Medicinal Plants Research. 2011;5(32):7022-30.
26. Garcia-Orozco KD, Sanchez-Paz A, Aispuro-Hernandez E, Gomez-Jimenez S, Lopez-Zavala A, Araujo-Bernal S, et al. Gene expression and protein levels of thioredoxin in the gills from the whiteleg shrimp (Litopenaeus vannamei) infected with two different viruses: The WSSV or IHHNV. Fish & Shellfish Immunology. 2012;32(6):1141-7.
27. Sak K, Nguyen TH, Ho VD, Do TT, Raal A. Cytotoxic effect of chamomile (Matricaria recutita) and marigold (Calendula officinalis) extracts on human melanoma SK-MEL-2 and epidermoid carcinoma KB cells. Cogent Medicine. 2017;4(1):1333218.
28. Pettit GR, Hoard MS, Doubek DL, Schmidt JM, Pettit RK, Tackett LP, et al. Antineoplastic agents 338. The cancer cell growth inhibitory. Constituents of Terminalia arjuna (Combretaceae). Journal of Ethnopharmacology. 1996;53(2):57-63.
29. Gálvez M, Martı, x, n-Cordero C, López-Lázaro M, Cortés F, et al. Cytotoxic effect of Plantago spp. on cancer cell lines. Journal of Ethnopharmacology. 2003;88(2):125-30.