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RESEARCH ARTICLE

Apoptosis induction by *Cassia angustifolia* leaf extract in breast cancer cell line and intracellular expression of inflammatory cytokine

Ehssan Nissiaf Jasim Al-Obaidy[1], Hamza N Hameed[2] & Nashwan Ibrahem Al-Lehebe[2*]

[1]Department of Biochemistry, Veterinary Medicine, Diyala University 32001, Iraq
[2]Department of Chemistry, College of Education for Pure Sciences, University of Mosul, Mosul 41001, Iraq

*Correspondence email - nashwan78ibrahem@uomosul.edu.iq

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Abstract

Cassia angustifolia is a famous and important medicinal plant around the world. It is used for the treatment of a lot of diseases to reduce inflammation in the skin and other parts of the body. Breast cancer has been distributed at a high rate in recent years among women and be most threatening to life. In this study the activity of *Cassia angustifolia* leaf extract was investigated as anticancer for breast cancer and immune response enhancement. WRL68, MCF7 and MDA-MB-231 cell line were treated with different doses of *C. angustifolia* leaf extract. Macrophage RAW 246.7 cell line was treated with *C. angustifolia* ethanol leaf extract for enhanced IL8, INF gamma and IL6 intracellular expression. Results showed that *C. angustifolia* ethanol leaf extract showed no toxic effect and did not affect the viability of WRL-68 liver cell line, while inhibiting the viability of breast cancer cell line MDA-MB-231 and MCF7 cell line a dose-dependent manner as presented. Also, influence on the intracellular expression of inflammatory cytokines as IL8, INF- gamma and IL6 were revealed enhanced significant the expression these cytokines within treated RAW cells with *C. angustifolia* leaf extract. To conclude, *C. angustifolia* ethanol leaf extract has anti-proliferation agent and enhancement of immune response by enhancing the intracellular expression of IL8, IFN-gamma and IL6 cytokines in the treated macrophage RAW cell line.

Keywords: breast cancer; *Cassia angustifolia*; IL8; IL6; INF-gamma

Introduction

Breast cancer has surpassed lung and prostate cancers as the most common cancer diagnosed worldwide. The frequency of breast cancer is rising worldwide, with 364000 cases predicted in the US by 2040 (1). Breast cancer is therefore a significant public health concern, as are the problems and therapies related to it. The stage and nature of the tumor determine the exact course of treatment for breast cancer, but in general, multidisciplinary breast cancer therapy consists of chemotherapy, radiation and surgery (2). Breast surgeons with training in general surgery or surgical oncologists usually perform surgical techniques for the treatment of breast cancer. Breast-conserving surgery (BCS), often known as a lumpectomy and mastectomy, which involves the more thorough removal of all breast tissue, are two types of breast cancer surgery (3). A new reality where survival after a breast cancer diagnosis is favorable has been brought about by advancements in diagnostic and treatment methods over the past few decades. According to estimates, there are 2.5 million breast cancer survivors living in the US and millions more around the globe 2. Surgery, radiation therapy, chemotherapy, hormonal therapy and other targeted therapies are all part of the curative care for breast cancer (4, 5).

The effectiveness and tolerance of several active substances (or their semi-synthetic counterparts) obtained from medicinal plants in the treatment of breast cancer have been evaluated (6). First-line or second-line treatments, including those for patients who are anthracycline-resistant, can employ medicinal plant-derived antitumor drugs alone or in combination. therapy for breast cancer that has spread or is confined (7).

Recently, medicinal plants have been used to treat many diseases. *Cassia angustifolia* is a famous and widely used medicinal plant around the world. It has been used for the treatment of a lot of diseases to reduce inflammation in the skin and parts of the body (8, 9). Moreover, it has demonstrated a remarkable antimicrobial agent (10, 11). Various parts of *C. angustifolia* have been utilized in traditional medicine due to its bioactive components, particularly flavonoids and phenols, which demonstrate anti-inflammatory, hepatoprotective, antifungal, antibacterial and antioxidant, as shown in Table 1 (12, 13). Furthermore, alkaloids extracted from this plant have also shown promise in combating bacteria and acting as antioxidants (14, 15). Among the phytoconstituents of *C. angustifolia*, rhein has antibacterial action, β-sitosterol has anticancer potential and sennoside is known for its laxative qualities (16).

Despite the development of numerous chemotherapeutic and radiological treatments, these treatments frequently have unfavorable side effects, such as immunosuppression and harm to healthy tissues(17, 18). This is demonstrated by the fact that about 74 % of novel anticancer drugs are either natural products or derived from natural products (19). Consequently, natural compounds derived from medicinal plants have gained increasing study as anti-cancer and anti-proliferative agents. Plant-derived nutraceuticals such as vitamins, alkaloids, polyphenols and flavonoids are emerging as new frontiers in the prevention and treatment of breast cancer and in suppressing cancer cell proliferation (20). To minimize adverse effects and enhance therapeutic outcomes, researchers are increasingly exploring the potential of these nutraceuticals as complementary therapies for breast cancer (21). The main aim of this study is to assess the effect of *C. angustifolia* leaf extract's cytotoxic capabilities on breast cancer cell lines (MCF-7 and MDA-MB-231), with an emphasis on its ability to induce apoptosis.

Materials and Methods

Cassia angustifolia leaf extracts preparation

The leaves of *C. angustifolia* were obtained from a local market in Tikrit, Iraq. The leaves were cleaned by mixing with distilled water in 1:1 ratio. The mixture was frozen and thawed 3 times, then treated with ultrasound instruments for 30 min (26). It was then filtered and dried using a lyophilizer to obtain the crude extract (27), then, grinding after that, used 100 g of leaf powder and immersed in 90 mL of 95 % ethanol for the preparation extract (23, 28).

Cell viability assay

C. angustifolia ethanol leaf extract was investigated for its anti-cancer ability for MDA-MB-231 and MCF7 breast cancer cell lines and MCF-10A normal cell line was obtained from the American Type Culture Collection (ATCC: Rockville, MD, USA).

Cells were cultured in DMEM supplemented with 10 % FBS and 1 % antibiotics (100 U/mL penicillin) in an incubator at 37 °C with 5 % CO_2. Cells were seeded into 96-well plates at densities of 2×10^3/well under 5 % CO_2 at 37 °C for 24 hrs (29). The cells were then treated with different concentrations of *C. angustifolia* ethanol leaf extract 125, 250, 500 and 1000 µg/mL. After 72 hrs incubation, 20 µL of MTT solution (5 mg/mL) was pipetted into each well and incubated for another 4 hrs. The medium was later discarded and the formazan precipitate was dissolved in DMSO. The absorbance of the mixtures was determined using a microtiter plate reader at 570 and 630 nm (background) and the cell viability was expressed as a percentage of live cells relative to controls. All experiments were performed in triplicate (30).

Diagnosis of the expression of inflammatory markers

The effect of *C. angustifolia* ethanol leaf extract on the expression of inflammatory markers such as IL8, IL6 and INF-gamma in the macrophages RAW 264.7 cell line (from Raybio C-series angiogenesis, USA) was evaluated. RAW 264.7 cells (1×106 cells/mL) were cultured for 24 hrs at 37 °C with 5 % CO_2 in a full DMEM medium (31). Following treatment with 100 µg/mL of *C. angustifolia* leaf extract, the cells were incubated for 6 hrs to examine the impact on the intracellular expression of inflammatory cytokines IL8, INF-gamma and IL6 as previously reported (23, 32). The BD Cytofix/CytopermTM kit and flow cytometry (Catalogue No. 555028) were used to measure the intracellular levels of these inflammatory markers.

Statistical analysis

Data were present by Mean (n = 3) per plate ± SD, ANOVA test used in the get the result, P value at ≤ 0.05 depend as significant value (33). The GraphPad Prism software was used to conduct statistical analysis tests.

Results

The percentage of breast cancer distribution is 11.7 % through the world and poses a critical threat to women (21). The type of triple-negative breast cancer is more aggressive and is hardly treated. Chemotherapy and radiotherapy have many sides effect on all body systems, especially the immune system, for that need to investigate a new treatment with fewer side effects and more effective as an anticancer. In recent years, researchers have been trying to find anticancer drug from natural sources as medicinal plants. In this study was investigated the ability of *C. angustifolia* ethanol leaf extract as an anti-proliferation agent and enhancement of immune response by effect on the breast cancer MCF7 and MDAMB231 cell line and the intracellular expression of inflammatory cytokines in the treated macrophage RAW cell line.

The effect of *C. angustifolia* ethanol leaf extract on MCF-7 and MDA-MB-231 breast cancer

The GC-MS analysis of *C. angustifolia* identified 11 active compounds as follows: tricosane, oxadecanoic acid, hexadecanoic acid, heptadecanoic acid, hexadecyl propyl ether, undecanal, octadecanoic acid, phthalic acid, carbonic acid, 2-pentadecanone and oxalic acid (23). Fig. 1 represents the cell viability (%) of MCF-7 and MDA-MB-231 breast cancer cell lines and WRL-68 normal liver cells after 24 hrs exposure to *C. angustifolia* ethanol leaf extract at (0, 125, 250, 500 and 1000 µg/mL). In general, *C. angustifolia* ethanol leaf extract exhibited no toxic effect and the viability of WRL-68 liver cell line remained above 95 % at all concentrations. In comparison to untreated cells, a significant reduction in the viability of MCF-7 cells to 48.2 %, 51 %, 59.8 % and 66.3 % and MDA-MB-231 to 42.2 %, 48.2 %, 53.4 % and 60.6 % at doses of 125, 250, 500 and 1000 µg/mL, respectively.

The current findings demonstrate that *C. angustifolia* ethanol leaf extract exhibits a dose-dependent cytotoxic effect against both MCF-7 and MDA-MB-231 breast cancer cell lines, with

Table 1. An overview of the principal findings and major pharmacological activities of *C. angustifolia* leaf ethanolic extracts

Biological activity	Principal findings study
Antioxidant and anticancer	A flavonoid-rich ethanol extract (such as rutin) demonstrated potent anti-MCF-7 and anti-HeLa cell activities (22).
Anti-inflammatory and immunomodulatory	RAW 264.7 cells produced less NO and GC-MS identified potent anti-inflammatory substances (23).
Antifungal	Strong antifungal activity, successfully preventing *Candida albicans* and *Candida auris* from growing *in vitro* (24).
Lipid profile and cardioprotective	Elevated HDL levels in Wistar rats without causing any negative cardiac consequences (25).
Antidiabetic (α-glucosidase inhibition)	Sennoside B was present in the ethanol extract demonstrated a moderate inhibition of the α-glucosidase enzyme(22).

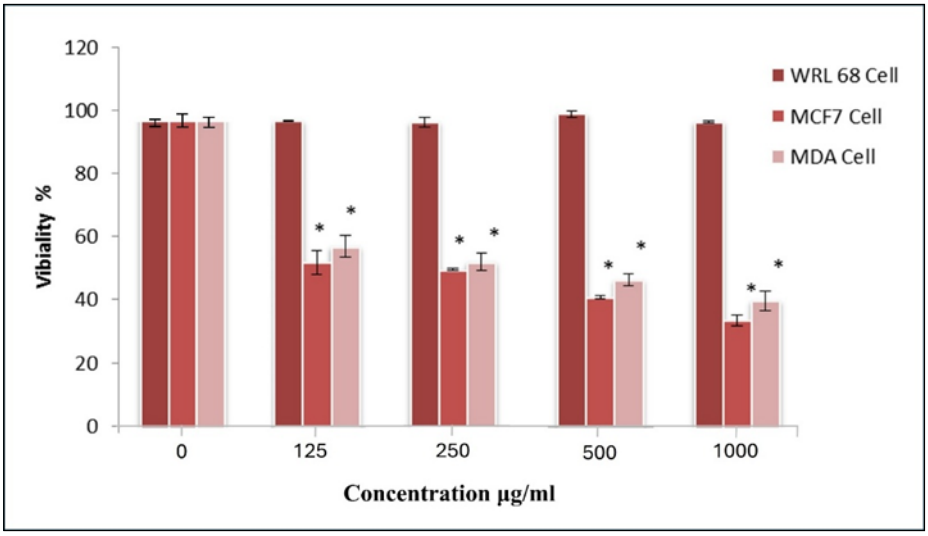

Fig. 1. The viability percentage (mean ± SD) effect of *C. angustifolia* ethanol leaf extract (C L) for 72 hrs on the viability of WRL-68 liver cell line and breast cancer cell line MDA-MB-231 and MCF7 cell line. *Significant at * P < 0.05. The mean (n = 3) ± SD for triplicate is used to express all values.

a more pronounced inhibition observed in MCF-7 cells.

The effect of *C. angustifolia* ethanol leaf extract on intracellular expression of inflammatory cytokines

In this study, we specifically examined how the *C. angustifolia* ethanol leaf extract (at a concentration of 100 µg/mL) affects immune signals in RAW 264.7 macrophages. As shown in Table 2 & Fig. 2, the extract significantly boosted the production of several important immune signaling molecules IL-8, IFN-gamma and IL-6 inside the treated RAW macrophage cells ($P < 0.05$). The extract triggered a remarkable increase in all 3 of these immune signals, with IL-8 showing the most impressive response, jumping nearly 200 times higher than normal levels. We saw similarly striking increases in both IFN-gamma and IL-6 production.

Discussion

The findings aligned with previous research which showed that the root of *C. daturic* had inhibitory effects on MDA-MB-231 and MCF-7 breast cancer cells, with IC50 values of 225.0 and 183.7 µg/mL, respectively (34).

Medicinal plants have many active compounds, such as antioxidants, polyphenols, flavonoids, vitamins and alkaloids, which affects the reduction of the severity of breast cancer (35) and also enhance the immune response by activating lymphocyte B cells through elevation of NFkB (7) and also influence many cases, such as epilepsy (36).

The therapeutic impact of these plant extracts is influenced by numerous factors such as the type of plant species, the part of the plant used, the extraction solvent and the origin of the plant material, which play crucial roles in determining the bioactivity of plant extracts (37). For instance, extracts of the leaves and flowers of *C. angustifolia* have demonstrated potent antioxidant qualities, including rutin, epicatechin and kaempferol derivatives, including kaempferol 3-O-sulphate-7-O-arabinopyranoside. These extracts may be the cause of the post-treatment reduction in breast cancer cell viability (MCF-7 and MDA-MB-231) (38, 39). Furthermore, *Nigella sativa* methanolic extracts exhibited significant cytotoxicity in MCF-7 cells but no cytotoxicity in MDA-MB-231 cells (40). Similarly, the *Ardisia crispa* hydromethanolic and ethyl acetate extracts exhibited weak activity against MDA-MB-231 cells and moderate cytotoxicity against MCF-7 cells, indicating that the cytotoxic response may differ depending on the cancer cell lines' classification and degree of malignancy (41, 42). In addition, *Mangifera indica* extract has been shown to suppress breast cancer by encouraging apoptosis and modifying the expression of mRNA linked to oxidative stress enzymes (43). *Cassia occidentalis* has been demonstrated to have anti-angiogenic and cytotoxic properties for MCF-7 cells (44). The balance between pro-angiogenic and anti-angiogenic factors such as transforming growth factor (TGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) plays a critical role in regulating tumor growth and progression (45).

Notably, research consistently shows that the levels of IL-6, IL-8 and IFN-γ are increased in immune cells, even though they are frequently linked to tumor progression according to the cellular context. In cancer tissues, these cytokines often correlate with poor prognosis and aggressive tumor, while in immune cells like RAW macrophages, their elevation may reflect a beneficial immunostimulant. Previous studies have revealed that inflammatory signals play a fascinating dual role in cancer biology. IL-6 and IL-8 induced chronic inflammation, specifically through enhanced angiogenesis and invasion, is essential to tumor development and metastasis (46). However, the context-dependent

Table 2. Intracellular expression of IL8, INF-gamma and IL6 on treated and untreated of RAW264.7 with *C. angustifolia* leaf ethanol extract using flow cytometry analysis

Cytokine	Untreated RAW Cells	Treated RAW Cells (100 µg/mL of *C. angustifolia* leaf ethanol extract)
IL-8	5.33 ± 2.08	1013.33 ± 46.19 *
IFN-γ	30 ± 7.94	306.33 ± 157.04 *
IL-6	9 ± 1	248 ± 82.27 *

(* indicates statistically significant increase)

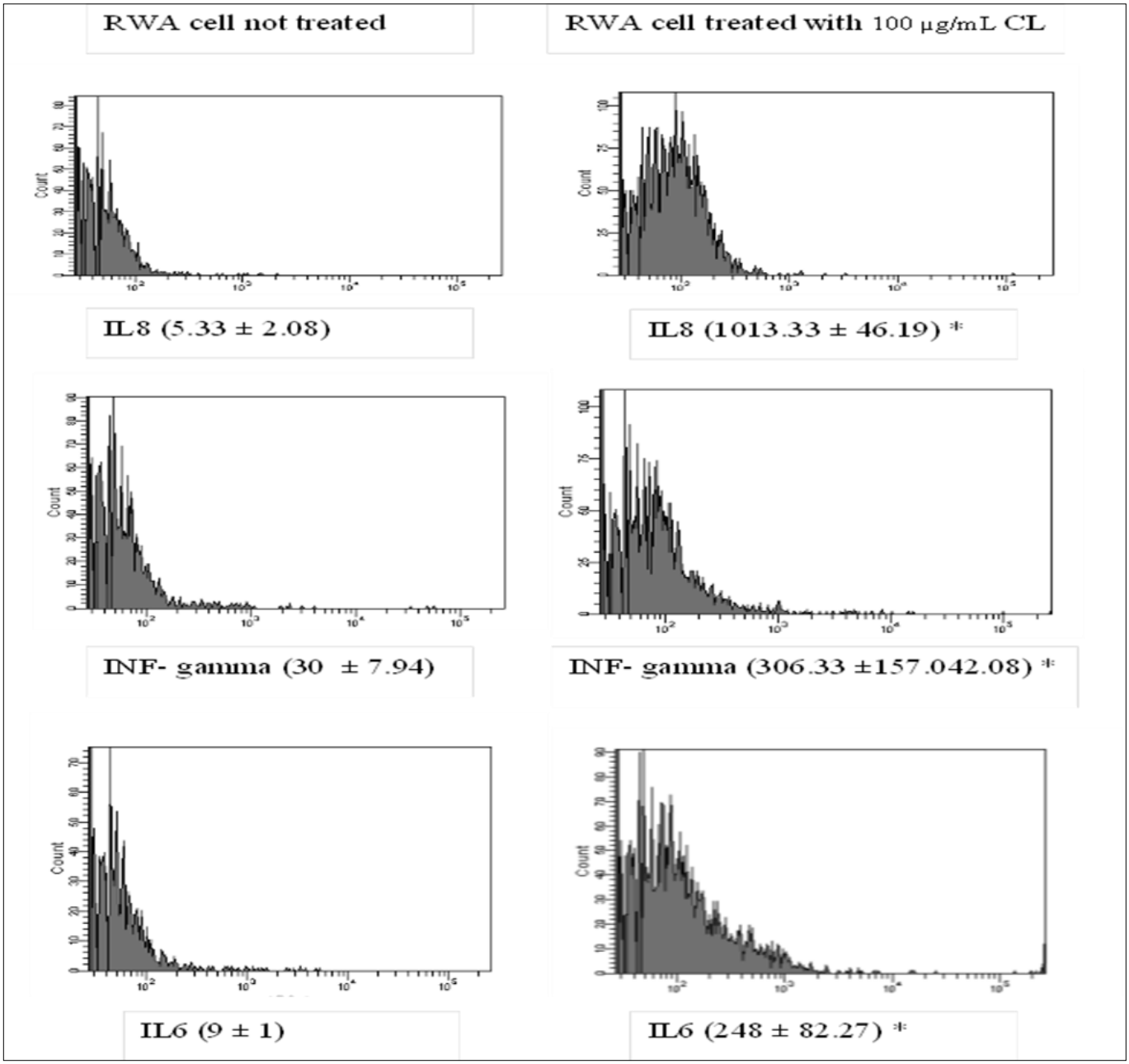

Fig. 2. Intracellular expression of inflammatory cytokines of IL8, IFN-gamma and IL6 presented as mean ± SD on RAW 264.7 cells. RAW 264.7 cells were split into two groups: cells treated with 100 µg/mL of *C. angustifolia* ethanol leaf extract (CL) for 24 hr (right panels) and cells left untreated (left panels). *Significant at $P < 0.05$.

nature of these cytokines (47). Similarly, the IFN-γ has dual functions, either boosting anti-tumor immunity or aiding immune suppression, depending on the tumor's microenvironment (37).

According to our results, *Cassia angustifolia* extract could potentially serve 2 important roles in breast cancer treatment: either promote anti-tumor immunity or contribute to immune suppression.

Conclusion

The ethanol leaf extract of *C. angustifolia* demonstrates as anti-proliferation agent and enhancement of immune response by affecting the breast cancer MCF7 and MDAMB231 cell line. Additionally, the extract markedly enhances the intracellular expression of IL8, INF-gamma and IL6 cytokines in the treated macrophage RAW-cell line.

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Authors' contributions

All authors contributed equally. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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