Advances in Biology & BioMedicine

Anveshika
Publishing & Informatics

RESEARCH ARTICLE

Anticancer effect of supercritical CO_2 extract of mango ginger (*Curcuma amada Roxb.*) in human breast cancer cells in vitro

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ABSTRACT

Breast cancer is the most common cancer in women accounting for about 1 out of 3 cancer cases in the US and 41760 deaths. Most of the metastatic breast cancers that are negative for progesterone, estrogen and HER2/neu are often aggressive and non-curable. Integrative treatment modalities combining complementary and alternative medicines along with conventional chemotherapeutics would be beneficial for improving the therapeutic efficiency and quality of life for breast cancer patients. We have investigated on the anticancer effects of supercritical CO_2 extract of mango ginger (*Curcuma amada* Roxb. - CA) in breast carcinoma cells (MCF-7) as well as transformed pre-malignant (MCF-10A) cells and analysed the molecular pathways affected by CA. CA is highly cytotoxic to MCF-7 cells as compared to MCF-10A cells with significantly lower IC values. CA also inhibited glycolysis by reducing the production of both lactate and ATP, the rate of inhibition being higher in MCF-7 cells than MCF-10 cells. The anticancer effect of CA in breast cancer cells is also demonstrated by the induction of apoptosis in MCF-7 cells as compared to MCF-10A cells. Analysis of biomarkers revealed that CA down regulated anti-apoptotic (p53 and Bcl-2), pro-metastatic (MMP-2, MMP-9), pro-inflammatory (COX-2) genes and up regulated pro-apoptotic (Bax, p21 and caspase-3), anti-metastatic (TIMP1) genes in MCF-7 breast cancer cells. These results indicated the anti-cancer effect of CA which could be further investigated for its use in breast cancer treatment.

KEYWORDS:

breast cancer, mango ginger, apoptosis, glycolysis, gene expression

HISTORY
Received :13 February 2020
Accepted :10 April 2020
Published :12 June 2020

INTRODUCTION

The prevalence of cancer continues to grow annually with approximately 18.1 million cases reported in 2018 with about 9.6 million cancer death (Bray et al., 2018). Among the most common cancers is breast cancer (about 11.6% of all types of cancer) with 2.1 million new cases per year and the second most common cancer after lung cancer in the world (Ferlay et al., 2015; Bray et al. 2018). However, the incidence of breast cancer is variable throughout the world with the highest rates occurring in the more developed countries (IARC and WHO, 2018). It was estimated that 626,679 cases of deaths (about 6.6% of all cancer deaths) were due to breast cancer in the world in 2018. Overall, breast cancer is the second cause of cancer death, with the standardized mortality rate (ASMR) of 12.9 (per 100,000), after lung cancer in the world and it is also the first cause of cancer death in women in most countries (Fitzmaurice et al., 2015; Bray et al., 2018). In the United States, excluding skin cancers, breast cancer is the most common cancer diagnosed among women accounting for nearly 1 in 3 cancers and 41,760 deaths (American Cancer Society, 2019; Siegel et al., 2019).

Breast cancer is also the most common cancer in women, and survivors with this diagnosis account for almost one fourth of the over 14 million cancer survivors in the US (Ghoncheh et al. 2016; Siegel et al., 2019). After several decades of preclinical and clinical research, much has been learned about the heterogeneity of breast cancer and has evolved a complex and multidisciplinary treatment approach to the disease. Although several treatment modalities are available, cure is an unrealized feature for the most aggressive forms of breast carcinomas such as triple negative (negative for progesterone, estrogen and HER2/neu) breast cancer (Jemal et al., 2008; Siegel et al., 2012; Siegel et al., 2019). Most triple negative breast cancers (as well as metastatic breast cancers are very difficult to treat and often non-curable. The main approaches to cancer management are often ineffective due to adverse reactions, drug resistance, or inadequate target specificity of single anti-cancer agents. Integrative treatment modalities combining complementary and alternative medicines along with conventional chemotherapeutics would be beneficial for improving the therapeutic efficiency and quality of life for breast cancer patients (Manson 2003; Chai et al., 2010; Prakash et al. 2013). We have reported on the anticancer and therapeutic effects of a patented supercritical CO_2 extract of Curcuma amada Roxb. (CA) that demonstrated substantial therapeutic efficacy against a variety of human cancers (Ramachandran et al., 2015a, b & c; Ramachandran et al., 2017). The supercritical CO_2 extract of mango ginger (CA) not only demonstrated significant cytotoxicity alone, but also showed synergistic cytotoxicity with conventional synthetic cancer drugs such as temozolomide, etoposide,

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vinblastine, etc in glioloblastoma and rhabdomyosarcoma cell lines (Ramachandran et al., 2015a, b and 2017). Recently we have demonstrated that CA has anti-glycolytic effects in cancer cells and can be combined with synthetic glycolytic inhibitors such as 2-deoxy-D-glucose and sodium oxamate (Ramachandran et al. 2018). Furthermore, CA has shown significant tumor inhibitory effects and therapeutic efficiency with irinotecan against glioblastoma nude mice xenografts (Ramachandran et al., 2017). In co-cultures of breast carcinoma cells with cancer associated fibroblasts, CA was shown to inhibit both Warburg and reverse Warburg effects thereby inhibiting the glycolytic pathway in both tumor cells and tumor microenvironment resulting enhanced therapeutic efficiency (Ramachandran et al., 2019). In the present investigation we report on the anticancer effects of CA in breast carcinoma cells (MCF-7) as well as transformed pre-malignant (MCF-10A) cells and analyse the molecular pathways affected by CA.

MATERIALS AND METHODS

Cell Culture

Human breast carcinoma (MCF-7) and pre-malignant breast cell lines (MCF-10A) were purchased from American Type Culture Collection, Manassas, VA and the cells were grown in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO_2 incubator. While MCF-7 has been reported to form tumors in nude mice, MCF-10A failed to develop tumors in it (Soule et al., 1990).

Supercritical CO_2 extract of mango ginger (Curcuma amada Roxb.)

Supercritical CO_2 extract of mango ginger (CA) was prepared by Flavex Naturextrakte GmbH, Rehlingen, Germany. The usual yield of CA extract was 2.5-3% of dried rhizome. The product has a brownish color and contains 10.2% of steam volatile components. Quantitative analysis by HPLC and GC-MS showed the presence of 61.7% (E)-labda-8(17),12diene-15,16 dial (LDD), 5.6% beta myrcene, 0.8% beta pinene, 0.3% ocimene, 0.2% beta cariophyllene besides other essential oil components in trace amounts. The chemical fingerprint details of CA have been described in our earlier publication (Ramachandran et al., 2015a).

Cytotoxicity assay

Breast carcinoma (MCF-7), pre-malignant breast cells (10000 cells/100 µl/well) were treated with increasing concentrations of CA in DMEM for 72 h in 96 well plates. MTT [3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay performed with the Cell Proliferation Kit I (Roche Biochemicals, IN) was used to analyze cytotoxicity of CA. The experiments were repeated four times with three replications for each treatment and the IC_{50}, IC_{75} as well as IC_{90} values were calculated from absorbance readings (Ramachandran et al., 2015b; 2017).

Apoptosis assay

MCF-7 and MCF-10A cells (10^3 cells/100 µl) were plated in 96-wells and treated with increasing concentrations of CA (0-100 µg/ml) for 24 h. Apoptosis induced by CA was analyzed using RealTime Glo Annexin V Apoptosis assay kit (Promega , Madison, WI) according to the manufacturer's protocol. Assay reagent (100 µl) containing Annexin-V-LgBiT (1X), Annexin-SmBiT (1X), CaCl2 (1X) and Annexin V NanoBiT substrate (1X) was added into each wells and shaken for 30 seconds on a plate shaker at 500-700 rpm to mix. Aliquots of 150 µl medium was transferred to an opaque 96-well plate and luminescence was measured in a Veritas luminometer. The luminescence readings were subtracted from the untreated sample and average relative luminescence readings (RLU) were plotted against CA concentrations.

ATP inhibition

MCF-7 and MCF-10A cells ($5x10^3$/100 µl medium/well) were plated in 96-well plates and incubated overnight at 37°C in a CO_2 incubator. Co-cultures were also grown by mixing MCF-7 cells with CAFs, normal fibroblasts or adipocytes in 1:1 ratio. On the next day, cells were treated with increasing concentrations of CA for 24 h in the CO_2 incubator. The plates were kept for 10 min at room temperature and 100 l of Cell Titer–Glo reagent (Promega Corporation, Madison, WI) was added into the wells and mixed well for 10 times. The plates were shaken on an orbital shaker for 10 min and kept at room temperature for another 10 min for stabilization of luminescent signal and 100 l of sample was transferred to a fresh 96-well plate before the plates were read in the Veritas Luminometer. The ATP in each well was calculated considering the ATP level in the control (untreated) as 100%. The experiment was repeated four times and the average values plotted (Ramachandran et al., 2019).

Lactate Inhibition

Both MCF-7 and MCF-10A cells ($1x10^5$/ 2 ml/well) were plated in 24-well plates and incubated overnight at 37°C in a CO_2 incubator. After 24 h of plating, cells were treated with increasing concentrations of CA followed by incubation at 37°C for another 24 h in a CO_2 incubator. About 0.5 ml medium was collected from each well for estimation of lactate and 1 ml of 8% perchloric acid was added into the media. The mixture was vortexed well, kept at 4°C for 5 min. and centrifuged for 10 min at 1500 x g. About 25 l of the extract was combined with the 1475 l of a working solution containing 10 mg nicotinamide adenine dinucleotide hydrate (NAD), 2 ml glycine buffer, 4 ml water and 100 units of lactate dehydrogenase for 30 min at room temperature. The reaction mix (200 µl) was transferred to 96-well plate and the plate was read at 340 nm within 10 min. The relative amounts of lactate in the

medium was calculated based on the lactate standard curve and plotted against drug concentrations (Ramachandran et al., 2018).

Western blot analysis

MCF-7 (3 x10^6/5 ml) were treated with increasing concentrations of CA (0, 10, 20, 50, 100 and 200 **µ**g/ml) for 72 h and total cellular protein was extracted using 0.5 ml of Invitrogen's protein extraction buffer (Invitrogen Corporation, Frederick, CA). The protein concentration was determined and 100 µg protein was separated on 10% SDS-PAGE. The separated protein was blotted on a nitrocellulose filter. The filters were hybridized with anti-human monoclonal/polyclonal antibodies specific for each Warburg effect- associated protein (MCT1, MCT4, HIF-1, Caveolin-1, and GLUT1 with β-actin control) in a western blot procedure and detected using the alkaline phosphatase color detection kit (Bio Rad Laboratories, Hercules, CA). The relative expression of proteins compared to untreated control samples were quantified using UNSCAN-IT gel™ software (Silk Scientific, Inc., Orem, UT). The relative increase or decrease in protein level was calculated based on untreated sample and fold-level changes were plotted against CA concentrations (Ramachandran et al., 2015a&b).

Statistical Analysis

Mean and standard deviation estimates were calculated using Microsoft Excel software using data from three separate experiments. The dose-dependent trends in relative protein expression was ascertained with samples treated with increasing CA concentrations. The relative protein expression levels (fold change) at different CA concentrations were plotted against CA concentrations.

RESULTS

Cytotoxicity

The cytotoxicity of CA in both breast carcinoma cells and pre-malignant cells is presented in Table 1. CA is significantly more cytotoxic to MCF-7 breast carcinoma cells than pre-malignant breast cells. The IC_{50}, IC_{75} and IC_{90} values for CA in breast cancer cell line (MCF-7) was 38.3, 41.7 and 47.7 µg/ml compared to 72.5, 131.5 and 191.5 µg/ml for MCF-10A pre-malignant cell line.

Effect of CA on ATP synthesis

The inhibitory effect of CA on ATP synthesis in breast carcinoma (MCF-7) and pre-malignant transformed cell lines (MCF-10A) are presented in Fig. 1. Although dose-dependent inhibition was noticed in both types of cells, the inhibitory effect is significantly higher in MCF-7 than MCF-10A cell line especially at lower doses of CA (<50 **µ**g/ml). Almost complete inhibition of ATP was observed in both cell lines at 50 µg/ml dose.

Effect of CA on lactate synthesis

Inhibition of lactate production by CA in both MCF-7 and MCF-10 A cell lines are given in Fig. 2. The inhibitory effect of CA in MCF-7 cell line is significantly higher than in MCF-10A cell line. The dose-dependent inhibition of lactate production in MCF-7 cells is very sharp compared to MCF-10A cells. CA at 50 **µ**g/ml had a 95% inhibition of lactate synthesis compared to only 25% inhibition in MCF-10A cells.

CA-induced Apoptosis

In both MCF-7 and MCF-10A cells, CA induced apoptosis (Fig. 3). The level of apoptosis increased up to 20 µg/ml CA in a dose-dependent manner and then plateaued afterwards. With further increase in CA concentration the percentage of cells undergoing necrosis increased (data not shown), practically destroying all cells. A clear difference in CA-induced apoptosis was evident between breast cancer (MCF-7) and pre-malignant (MCF-10A) cells, with more cells in MCF-7 undergoing apoptosis than MCF-10A cells. This preferential killing of breast cancer cells compared to pre-malignant cells is an added advantage of CA.

The induction of apoptosis at four concentrations of CA (0, 5, 10 and 20 **µ**g/ml) and two time periods (6 h and 24 h) in both MCF-10A and MCF-7 cell lines are presented in Fig. 4. MCF-10A cells show significantly lower level of apoptosis than MCF-7 in both 6 h and 24 h treatment periods and at all CA concentrations.

Western blot hybridization

Fig. 5 presents the protein expression of apoptosis-associated genes in MCF-7 cells treated with increasing concentrations of CA in vitro. Western blots were scanned, quantified and the mean values of fold level decrease/increase over untreated control cells were plotted (Fig. 5a). The transcription factor STAT3 (Signaling transducer and activator of transcription 3) showed a dose-dependent inhibition with CA treatment. The pro-apoptotic genes p21, BAX and caspase-3 were up regulated with increasing doses of CA. On the other hand, the anti-apoptotic genes such as p53 and Bcl-2 were down regulated with increasing concentrations of CA (Fig. 5b).

Two genes associated with inflammation were also analyzed in CA treated MCF-7 cells (Fig. 6a & b). While COX-2(cyclooxygenase 2) showed a decreasing trend with increasing doses of CA, another marker iNOS (inducible nitric oxide synthase) were not much affected by it.

Four biomarkers associated with metastasis were also analyzed in CA-treated MCF-7 cells and the results are presented in Fig. 7a and 7b. While CA inhibited the two metalloproteinase genes (MMP2 and MMP9), it failed to show any significant change in VEGF-R proteins. Interestingly, the tumor inhibitor of metalloproteinase 1 (TIMP1) showed a significant upregulation by CA.

Table 1. Cytotoxicity of supercritical CO_2 extract of mango ginger (*Curcuma amada* Roxb.- CA) in MCF-7 and MCF-10A cell lines.

Cell line	IC_{50} (µg/ml)	IC_{75} (µg/ml)	IC_{90} (µg/ml)
MCF-7	38.3 ± 1.6	41.7 ± 1.1	47.7 ± 0.1
MCF-10A	72.5 ± 7.8	131.5 ± 6.4	191.5 ± 2.1

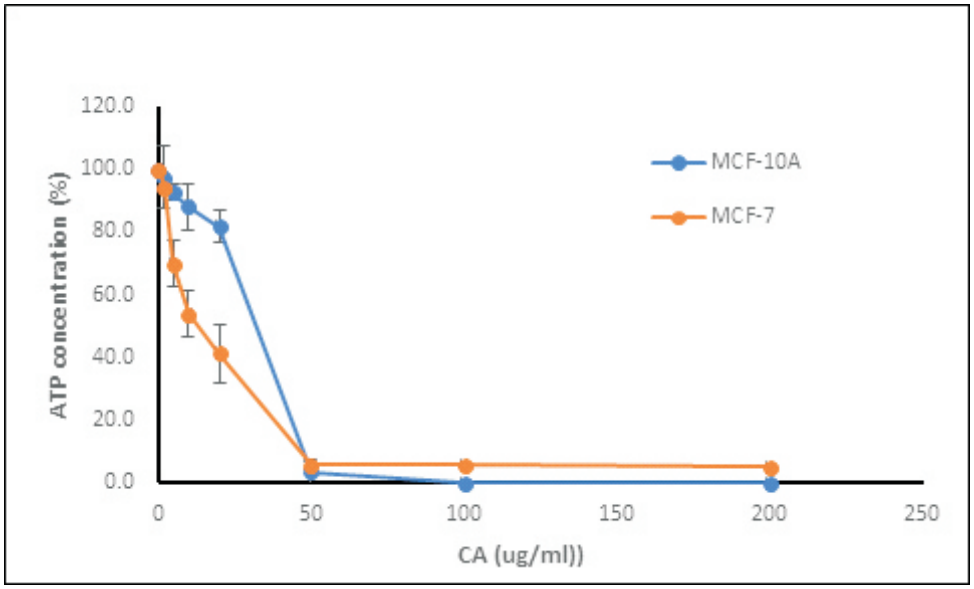

Figure 1. Effect of CA on ATP synthesis in MCF-7 and MCF-10A cell lines.

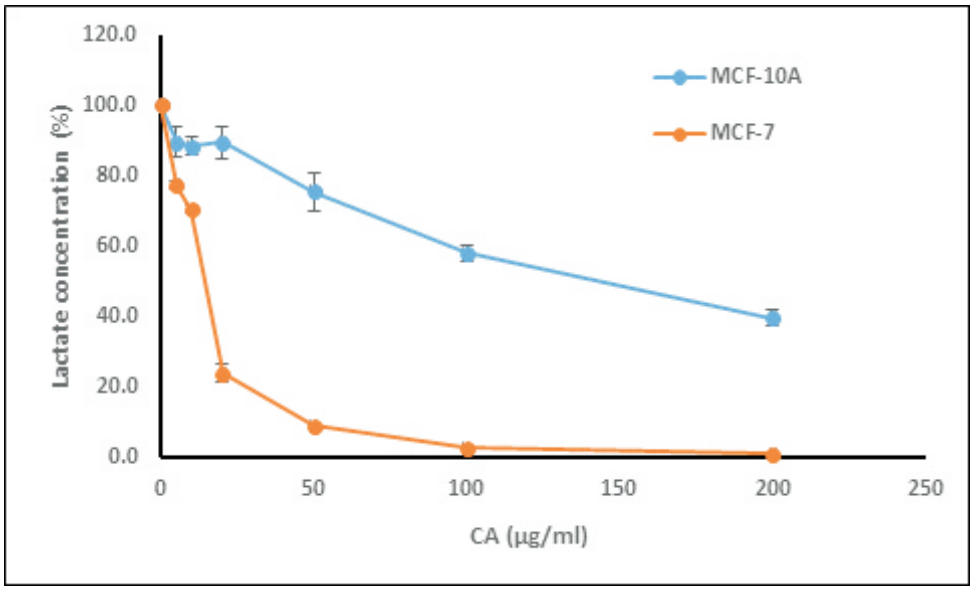

Figure 2. Effect of CA on lactate synthesis in MCF-7and MCF-10A cell lines.

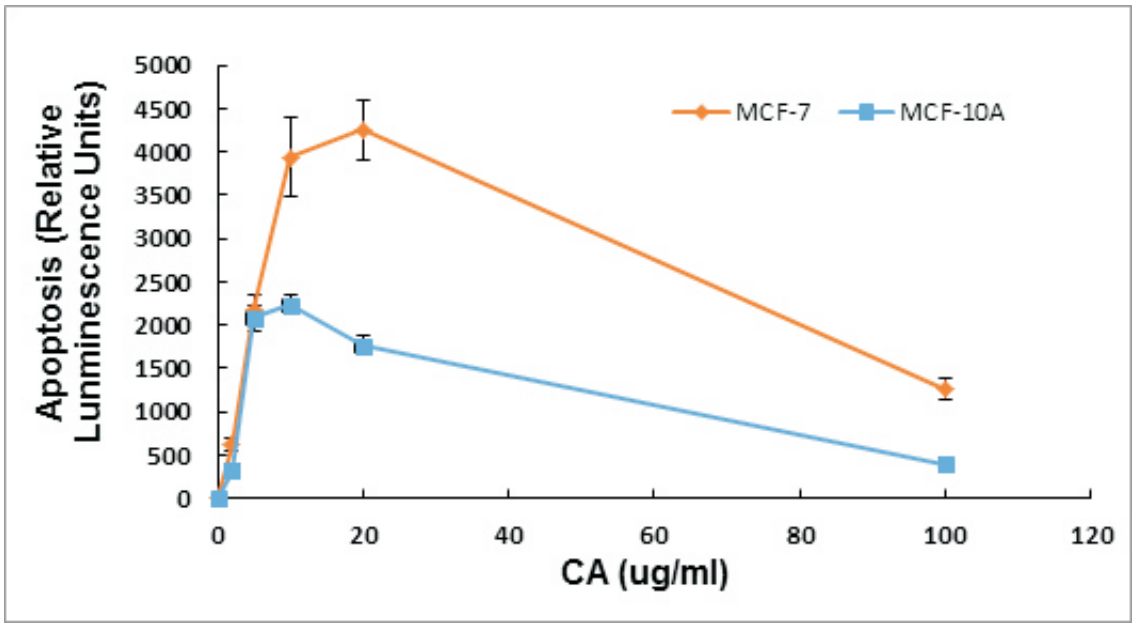

Figure 3. Analysis of CA-induced apoptosis in MCF-7 and MCF-10A cells. Cells were treated with increasing concentrations of CA for 24h and the level of apoptosis analyzed with RealTime Glo Annexin V Apoptosis assay kit (Promega , Madison, WI).

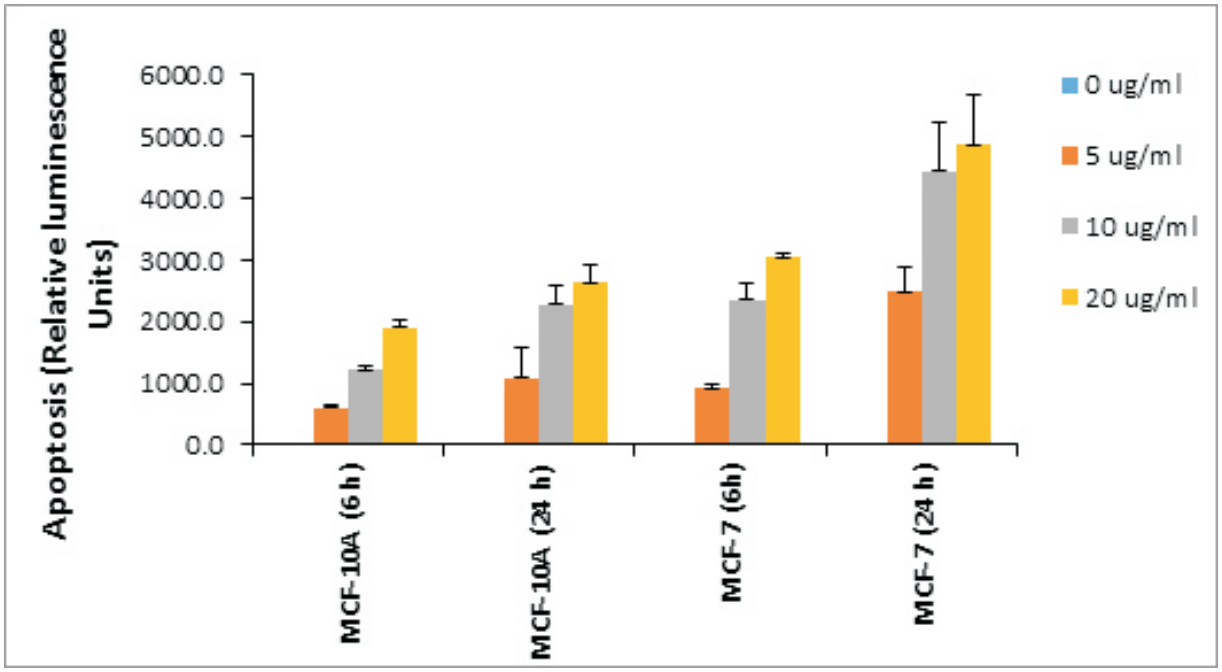

Figure 4. Apoptosis induced by 6 h and 24h of CA treatment (0-20 µg/ml) in MCF-7 and MCF-10A cells analyzed by RealTime Glo Annexin V Apoptosis assay kit (Promega , Madison, WI).

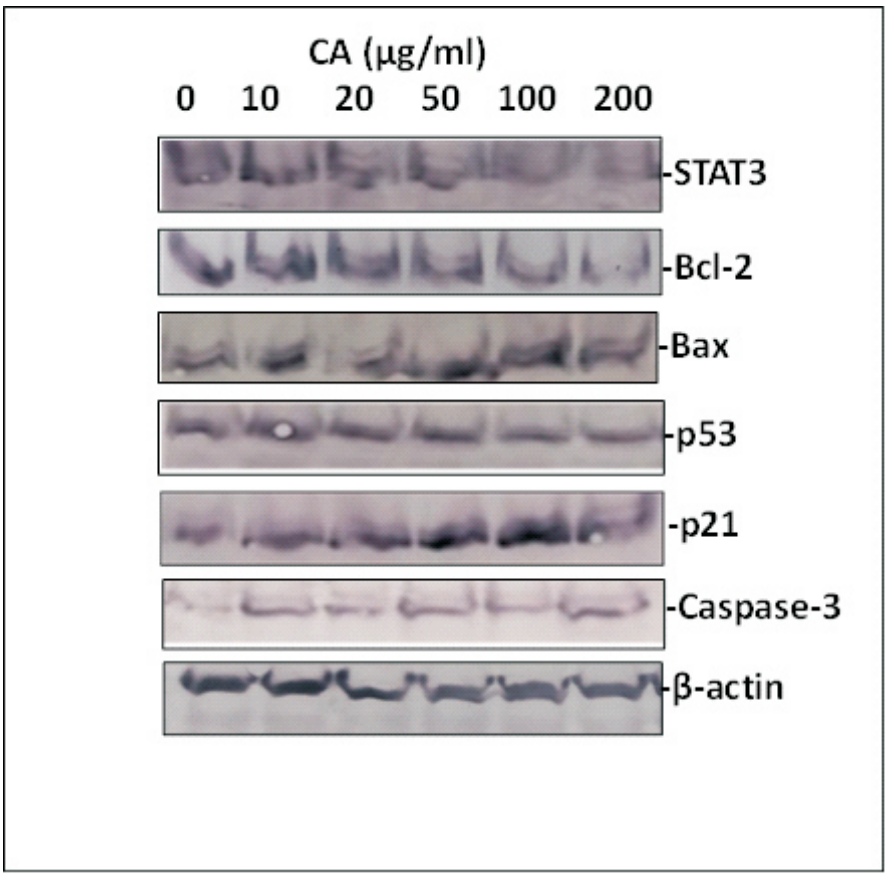

Figure 5a. Analysis of protein expression of apoptotic genes in MCF-7 cells treated with CA (0-200 µg/ml). Western blot hybridization was used for analysis of protein expression.

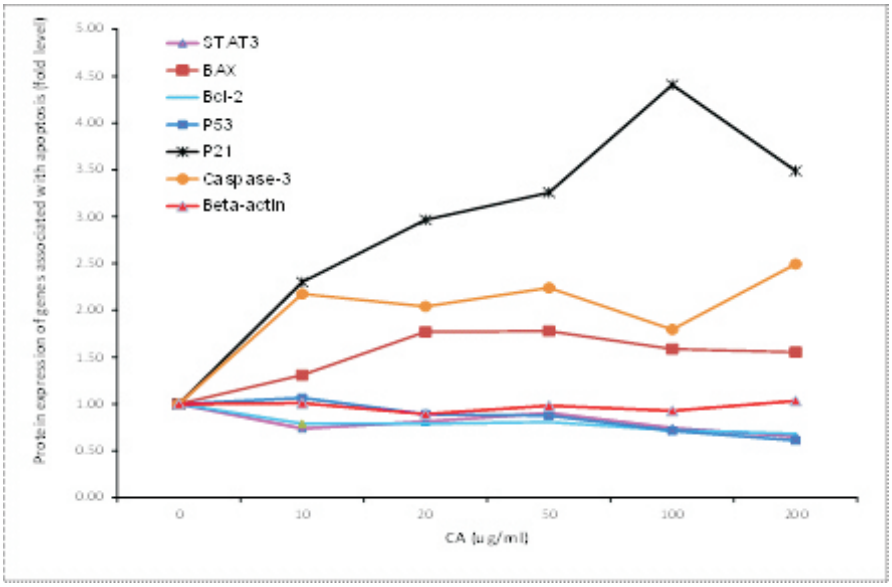

Figure 5b. Quantification of protein expression (fold level over untreated cells) of apoptotic proteins in CA-treated MCF-7 cell line. Mean protein expression at each concentration was plotted and the standard deviation values (not included) were less than 10% of mean values.

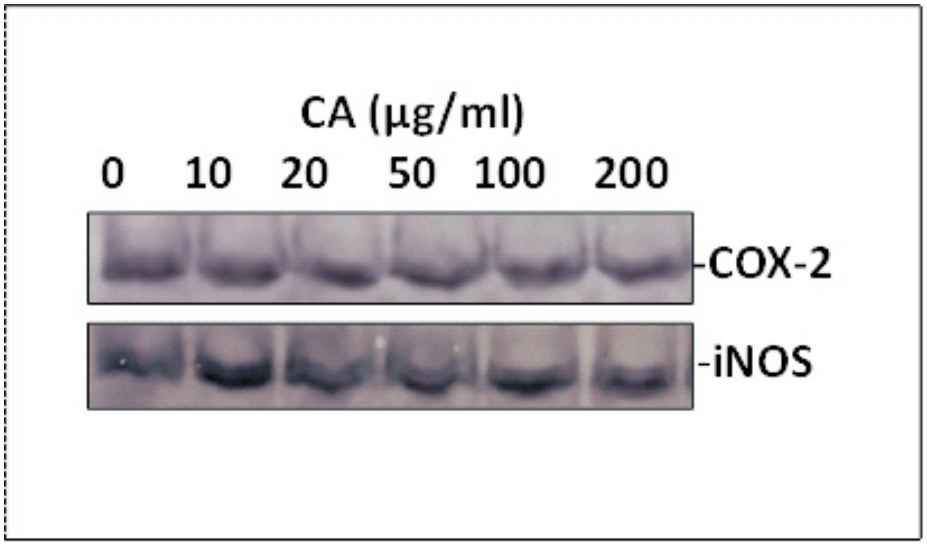

Figure 6a. Analysis of protein expression of two inflammatory genes (COX-2 and iNOS) in CA-treated MCF-7 cell line.

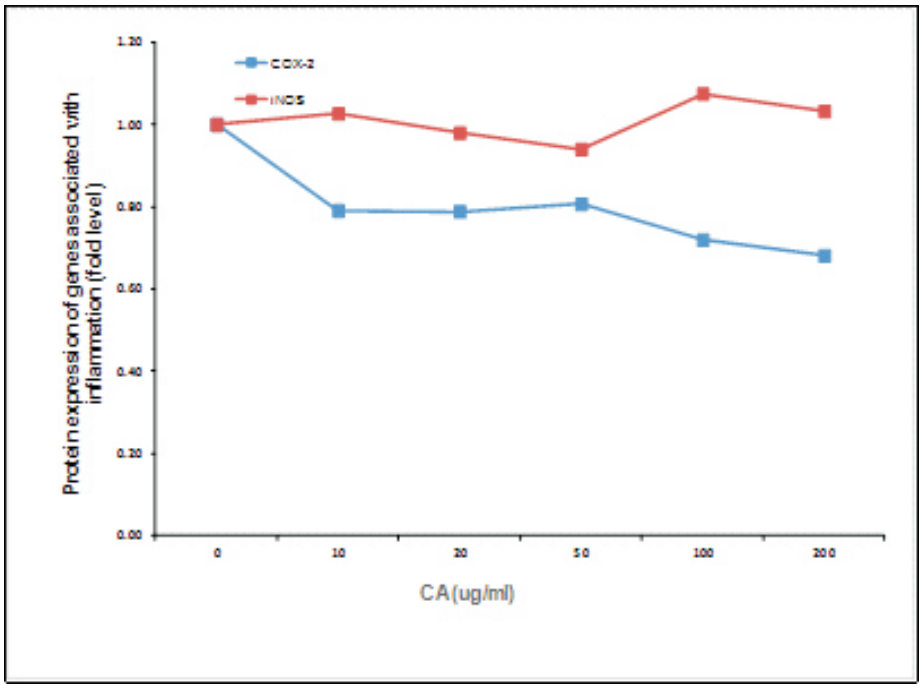

Figure 6b. Quantification of protein expression (fold level over untreated cells) of genes associated with inflammation in CA-treated MCF-7 cell line. Mean protein expression at each concentration was plotted and the standard deviation values (not included) were less than 10% of mean values.

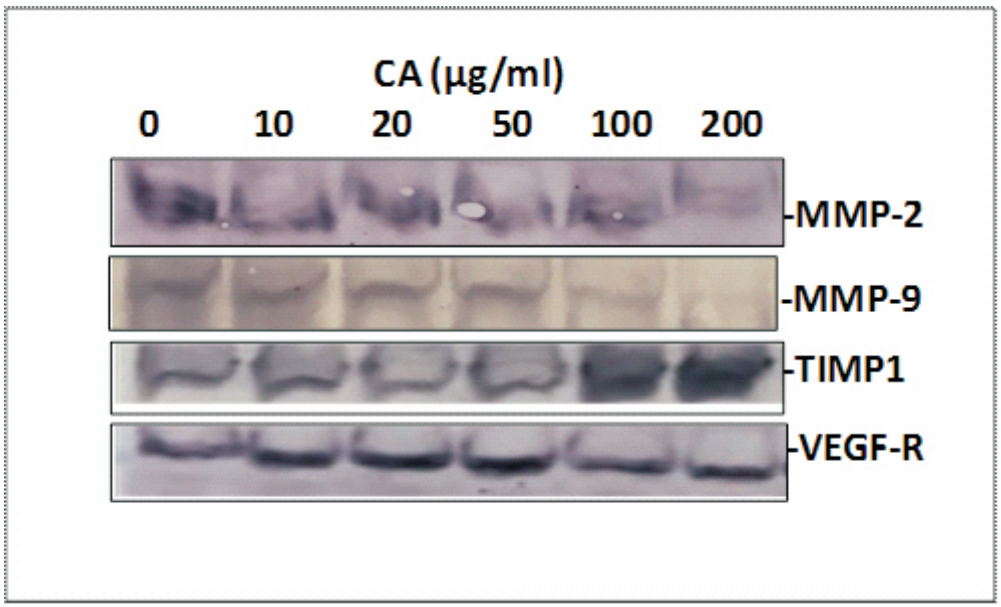

Figure 7a. Analysis of protein expression of genes associated with metastasis in CA-treated MCF-7 cell line.

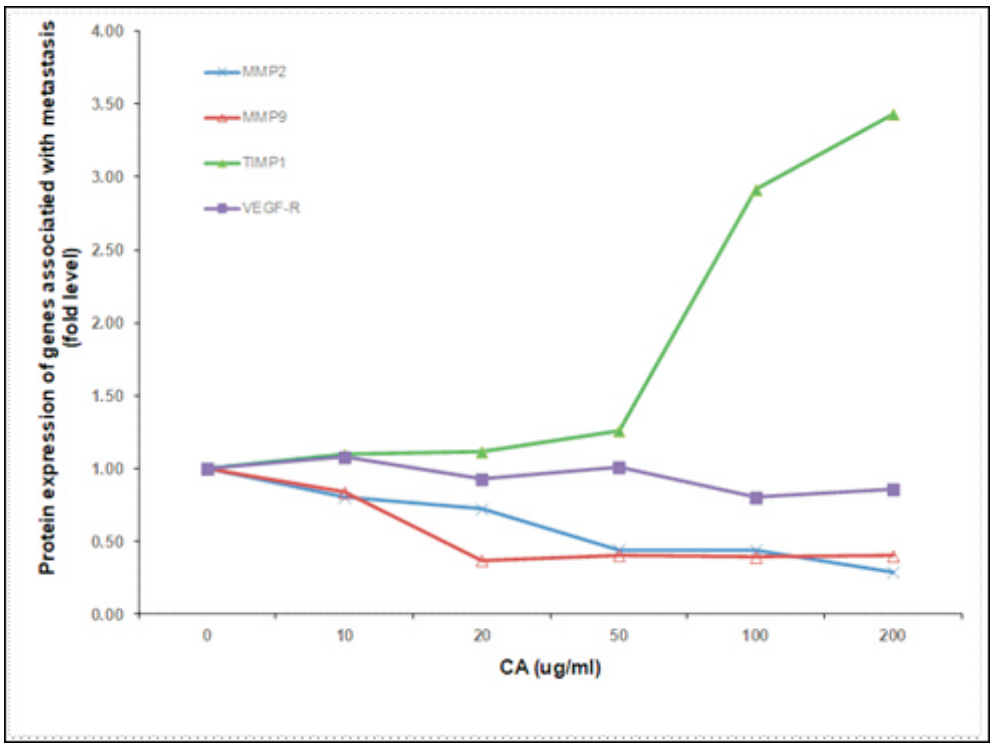

Figure 7b. Quantification of protein expression (fold level over untreated cells) of genes associated with inflammation in CA-treated MCF-7 cell line. Mean protein expression at each concentration was plotted and the standard deviation values (not included) were less than 10% of mean values.

DISCUSSION

Many approaches to cancer management are often ineffective due to adverse reactions, drug resistance, or inadequate target specificity of single anticancer agents. There is now emerging interest in developing drugs that mitigate these problems with compounds that affect multiple targets with reduced side effects and which are effective against several cancer types. Natural compounds from various sources including plants offer an important opportunity for discovery of novel therapeutic candidates for the treatment of cancer (Newman et al., 2003).

Cytotoxicity investigations in the present study indicated that CA showed exceptionally higher cytotoxicity against MCF-7 breast cancer cells than pre-malignant MCF-10A cells. In our previous studies we have noticed the specificity of CA towards malignant cells compared to normal cells in vitro (Ramachandran et al., 2019). We have also reported that CA can be combined with conventional cancer drugs such as temozolomide, etoposide, vinblastine, and cyclophosphamide (Ramachandran et al., 2015a &b). The major ingredient in CA is a dialdehyde viz., (E)-labda-(8,17)-12, diene-15,16-dial (LDD) which is about 61.7% of CA and it appeared to be responsible for the increased cytotoxity of CA in glioblastoma cells (Ramachandran et al., 2015a, b and c).

The cytotoxicity data also correlated with inhibition of ATP and lactate synthesis by CA, as it showed significantly higher inhibition of ATP and lactate in MCF-7 cells than MCF-10A cells.

Cancer cells demonstrate high uptake of glucose and are more dependent on aerobic glycolysis to produce ATP for growth and maintenance (Warburg, 1956; Brown, 1962; van der Heiden et al., 2009). Since therapeutic selectivity or preferential killing of cancer cells without significant toxicity to normal cells is one of the most important considerations in cancer chemotherapy, targeting the metabolic pathway offers the potential for a selective approach to cancer treatment. In the present investigation, inhibition of both lactate and ATP in breast carcinoma cells showed the anti-glycolytic effect of CA, thereby indicating the preferential targeting of cancer cells over normal cells (Ramachandran et al., 2018). Also in a recent report using a model of breast cancer environment, we described that CA can inhibit both conventional and reverse Warburg effects in tumor environment in vitro (Ramachandran et al., 2019).

Analysis of apoptosis showed that the degree of apoptosis induced by CA in breast carcinoma cells is significantly higher than in pre-malignant cells. CA was able to induce apoptosis in a time and dose-dependent manner. Breast cancer cells treated with CA at higher concentrations (>50 μg/ml) induces cell necrosis (data not shown) and cause complete cell death. Although the mechanisms and morphologic features of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the "apoptosis-necrosis continuum" (Zeiss, 2003). For example, two factors that will convert an ongoing apoptotic process into a necrotic process include a decrease in the availability of caspases and intracellular ATP (Leist et al., 1997; Denecker et al., 2001).

Drug-induced apoptosis in cancer cells follow one of the two major pathways classified as either intrinsic mitochondrial or extrinsic death receptor pathways (Kromer et al., 2007; Amaral et al., 2010; Fulda, 2010; Millimouno et al., 2014). In the mitochondrial pathway, death stimuli target mitochondria either directly or through transduction by pro and anti-apoptotic members of the Bcl-2 family including Bcl-2 and Bax (Dewson and Kluck, 2009). The mitochondria then release apoptogenic proteins, ultimately leading to caspase activation and apoptotic process. Several naturally occurring compounds target tumor cells by regulating cell death pathways such as extrinsic and intrinsic apoptosis pathways and autophagic pathways (Fulda, 2010).

Effective development of an anti-cancer drug needs to consider different sets of upregulated, downregulated, and mutated genes and their regulatory pathways in cancer cells. Protein expression analysis by western blot hybridization showed that apoptotic effect of CA in breast cancer cells correlated with the downregulation of anti-apoptotic genes like Bcl-2, mutant p53 and upregulation of pro-apoptotic genes like p21, Bax and caspase-3. Our previous investigations with CA have shown similar effects on apoptosis-associated genes in human rhabdomyosarcoma and glioblastoma cells in vitro and in vivo (Ramachandran et al., 2015a, b and 2017). CA also inhibited inflammatory biomarker protein Cox-2 and metastasis-associated proteins like MMP2 and MMP9 and upregulated TIMP1. This type of modulatory effects were also noticed earlier in glioblastoma and rhabdomyosarcoma cell lines (Ramachandran et al., 2015, 2019). In conclusion, the anticancer effect of CA in breast carcinoma is demonstrated and therapeutic use of this molecule can be further explored for breast cancer treatment.

CONFLICT OF INTEREST

The authors declare the following conflict of interest with respect to the research, authorship, and/or publication of this article. Dr. Steven J. Melnick is the founder of Dharma Biomedical LLC, which is an evidence-based ethnobotanical and evochemical drug discovery and nutraceutical company operating on a for-profit basis. Dr. Karl-Werner Quirin is the Chief Executive Officer of Flavex Naturextrakte GmbH, Rehlingen, Germany, a company producing specialty botanical extracts for cosmetics and food supplements on the basis of supercritical CO_2 extraction. Dr. Cheppail Ramachandran and Mr. Daniel Moy are employees of Dharma Biomedical LLC.

ETHICALAPPROVAL

This in vitro investigation did not involve any human subjects or live animals. Therefore, Institutional Review Committee (IRB) and Institutional Animal Care and Use Committee (IACUC) approvals were not applicable.

REFERENCES

[1]. American Cancer Society. Breast Cancer Facts and Figures 2019-2020. Atlanta, GA: American Cancer Society, 2019.

[2]. Amaral JD, Xavier JM, Steer CJ et al. The role of p53 in apoptosis. Discov Med 2010; 9: 145-152.

[3]. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68:394-424.

[4]. Brown J. Effects of 2-deoxyglucose on carbohydrate metabolism: review of literature and studies in the rat. Metabolism: clinical and experimental. 1962; 11: 1098-111

[5]. Chai, S.; To, K.; Lin, G. Circumvention of multi-drug resistance of cancer cells by Chinese herbal medicines. Chin. Med 2010; 5, 26.

[6]. Dewson G, Kluck RM. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. J Cell Sci. 2009,122 :2801-8

[7]. Denecker G, Vercammen D, Declercq W, et al. Apoptotic and necrotic cell death induced by death domain receptors. Cell Mol Life Sci 2001; 58:356–70.

[8]. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; **136**, E359-E86.

[9]. Fitzmaurice C, Dicker D, Pain A, et al. The Global Burden of Cancer 2013. JAMA Oncol, 2015; **1**, 505-27.

[10]. Fulda, S. Evasion of apoptosis as a cellular stress response in cancer. Int J Cell Biol 2010; 370835. doi: 10.1155/2010/370835

[11]. Ghoncheh M, Pournamdar Z, Salehiniya H. The incidence and Mortality and Epidemiology of Breast Cancer in the World.

[12]. International Agency for Research on Cancer (IARC) and World Health Organization (WHO). GLOBOCAN 2018: Breast..

[13]. Jemal A, Siegel R, Ward E, et al. Cancer statistics. CA Cancer J Clin 2008; 58:71–96.

[14]. Kromer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev 2012; 87: 99-163.

[15]. Leist M, Single B, Castoldi AF, et al. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997; 185: 1481–6.

[16]. Manson M.M. Cancer prevention–the potential for diet to modulate molecular signalling. Trends Mol Med 2003; 9, 11–18.

[17]. Prakash O, Kumar A, Kumar P. Anticancer potential of plants and natural products: A review. Am J Pharmacol Sci 2013; 1: 104–111

[18]. Millimouno FM, Dong J,. Yang L, et al. Targeting apoptosis pathways in cancer and perspectives with natural compounds from Mother Nature. Cancer Prev Res 2014; 7: 1081–1107.

[19]. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 2003; 66: 1022–1037.

[20]. Ramachandran C, Lollett I, Escalon E, et al. Anticancer potential and mechanism of mango ginger supercritical CO2 extract in human glioblastoma cells. J Evid Based Compementary Altern Med 2015a; 20: 109-119.

[21]. Ramachandran C, Quirin K-W, Escalon E, et al. Therpaeutic effect of supercritical CO2 extracts of *Curcuma* species with cancer drugs in rhabdomyosarcoma cell lines. Phytother Res 2015b; 29: 1152-1160.

[22]. Ramachandran C, Portalatin G, Quirin K-W, et al. Inhibition of AKT signaling by supercritical CO2 extract of mango ginger (Curcuma amada Roxb.) in human glioblastoma cells. J Complement Integr Med 2015c; 12: 307-315.

[23]. Ramachandran C, Portalatin GM, Prado A, et al. In vivo antiutmor effect of supercritical CO2 extract of mango ginger (Curcuma amada Roxb.) in U87-MG human gliobalstoma nude mice xenografts. J Evid Based Complementary Altern Med 2017; 22: 260-267.

[24]. Ramachandran C, Juan A, Quirin K-W, et al. Synergistic effect of supercritical CO2 extract of mango ginger (Curcuma amada Roxb.) with glycolytic inhibitors in human glioblastoma cells in vitro. Advances in Cancer Research & Therapy 2018; 3: 1-17.

[25]. Ramachandran C, Juan A, Moy D, et al. Metabolic synergy of breast cancer cells with tumor microenvironment: interaction of breast cancer cells with normal fibroblasts, cancer-associated fibroblasts and adipocytes in vitro. Advances in Cancer Research & Therapy 2019 (in press).

[26]. Siegel R, Naishadham D, Jemal A. Cancer statistics for Hispanics/Latinos. CA J Clin Cancer 2012; 62:283–98.

[27]. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin, 2019; 69:7-34.