RESEARCH ARTICLE

In Vitro Apoptosis Triggering in the BT-474 Human Breast Cancer Cell Line by Lyophilised Camel's Milk

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Abstract

Breast cancer is a global health concern and is a major cause of death among women. In Oman, it is the most common cancer in women, with an incidence rate of 15.6 per 100,000 Omani females. Various anticancer remedies have been discovered from natural products in the past and the search is continuing for additional examples. Cytotoxic natural compounds may have a major role in cancer therapy either in potentiating the effect of chemotherapy or reducing its harmful effects. Recently, a few studies have reported advantages of using crude camel milk in treating some forms of cancer. However, no adequate data are available on the lyophilised camel's milk responsibility for triggering apoptosis and oxidative stress associated with human breast cancer. The present study aimed to address the role of the lyophilised camel's milk in inducing proliferation repression of BT-474 and HEp-2 cells compared with the non-cancer HCC1937 BL cell line. Lyophilized camel's milk fundamentally repressed BT-474 cells growth and proliferation through the initiation of either the intrinsic and extrinsic apoptotic pathways as indicated by both caspase-3 mRNA and its action level, and induction of death receptors in BT-474 but not the HEp-2 cell line. In addition, lyophilised camel's milk enhanced the expression of oxidative stress markers, heme-oxygenase-1 and reactive oxygen species production in BT-474 cells. Increase in caspase-3 mRNA levels by the lyophilised camel's milk was completely prevented by the actinomycin D, a transcriptional inhibitor. This suggests that lyophilized camel's milk increased newly synthesized RNA. Interestingly,it significantly (p<0.003) repressed the growth of HEp-2 cells and BT-474 cells after treatment for 72 hours while 24 hours treatment repressed BT-474 cells alone. This finding suggests that the lyophilised camel's milk might instigate apoptosis through initiation of an alternative apoptotic pathway.

Keywords: Breast cancer - camel's milk - anticancer activity - zymography - Oman

Introduction

Cancer affects people all over the world (Burney et al., 2014). In 2008, the World Health Organisation (WHO) reported that cancer is one of the leading causes of 7.6 million deaths worldwide. In prosperous countries, about 20% or one in five people will die of cancer. Among the most common malignancies is breast cancer. In Oman, it is considered the commonest female cancer, accounting for one of every five cancers detected in females with an age standardized incidence rate (ASR) of 15.6 per 100,000 of the Omani female population(al-Lawati et al., 1999; Al-Hamdan et al., 2009). Cancer is an acquired genetic disease resulting from exposure to environmental carcinogens over many years (Levin et al., 2008) with a contribution of mutations in a single cell, which give the cell ability to grow uncontrollably and escape the normal controls of proliferation (Kittaneh et al., 2013).

During normal self-renewal, proliferation is an important and vital physiological process for cells to survive, which is required for maintenance of the cellular multi-potency and the power to regenerate (Millership et al., 2010; Fuchs and Chen, 2013). On the other hand, damaged DNA normally undergoes repair, however, if the process of repair does not take place, the unpaired DNA might lead to carcinogenesis (Abbotts et al., 2014). However, during the cell cycle and as a normal physiological process, cells undergo apoptosis. The regulation of apoptotic process is very important in the maintenance of homeostasis and the loss of the apoptotic ability of the cell is implicated in a wide variety of human diseases. This is initiated by a wide variety of either extrinsic or intrinsic signals as well as stimuli and hence critical in several disease processes (Korashy et al., 2012b). These signals instructing the cells to undergo, apoptosis through the activation of a family of proteins known as caspases. The intrinsic signals can

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initiate apoptosis through mitochondrial oxidative stress caused by free radicals (Elmore, 2007) that ultimately lead to neoplasia (Seyfried and Shelton, 2010). Studies utilizing transgenic and knockout small animal models provide direct confirmation that disruption of apoptosis can promote tumor development and metastasis (Seyfried et al., 2014).

The increasing incidence of cancer has led to a global burden to find many different therapeutic ways including chemotherapy and surgical procedures. The stage of the disease determines modality of treatment including surgery, radiation or chemotherapy. Many of these modalities are targeting signaling pathways responsible for proliferation of cell and survival (Domvri et al., 2013). For example, most of the clinically used cytotoxic anticancer drugs, such as doxorubicin, 5-Fluorouracil, and cisplatin, have the capacity to trigger apoptosis in susceptible cancer cells (Hassan et al., 2014a).

The combination of all these moderate modalities in treatment increases the therapeutic efficacy but might itself induces a potential risk of toxicity by the drug itself (Feng et al., 2014). Thus, one of the strategies for inhibition of cancer development incorporates attenuation of anti-apoptotic genes. Therefore, new chemo-preventive agents capable of inhibiting cell proliferation and inducing apoptosis in cancer cells with less or no side effects would be required. Hence, apoptosis remains the main target for cancer treatment by either naturally occurring or synthetic agents (Reed and Pellecchia, 2005).

Naturally occurring compounds have contributed to many roles in cancer chemotherapeutics. Many plants have been identified as a source of pharmaceutical products (Ivanova et al., 2005; Mothana et al., 2012; Ryu et al., 2012).Several natural compounds have shown anti-oxidant activity in various bioassay systems related to human disease(Aziz et al., 2003; Mothana et al., 2012). Furthermore, chemoprevention by dietary constituents in the form of functional food has an excellent role in health promotion and emerged as a novel approach to control cancers (Kontou et al., 2011) and other diseases (Agamy et al., 1992; Harrison et al., 2006; Hasson et al., 2010a, 2010b).

Additionally, the focus now is shifted towards animal and animal-products sources. Many animals have been used as a source for many therapeutic agents such as camel(Harrison et al., 2003) and crocodilians (Kommanee et al., 2012). Despite the fact that crocodilians are known to live with opportunistic bacteria they suffer no adverse effect. Recent studies showed that the plasma of crocodilians is a good source for an antibiotic agent against several pathogenic bacteria (Pata et al., 2011; Kommanee et al., 2012). Camels are another example of animal models with many products that have been used traditionally in treating some diseases such as hepatitis, asthma and problems of spleen (Harrison et al., 2003; Korish and Arafah, 2013). Late studies have reported that camel's milk (Harrison et al., 2003) is the most effective milk among the other ruminant milks against Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, and rotavirus Conesa et al., 'Isolation of Lactoferrin from Milk of Different Species: Calorimetric

and Antimicrobial Studies'; Althnaian, Albokhadaim, and El-Bahr, 'Biochemical and Histopathological Study in Rats Intoxicated with Carbontetrachloride and Treated with Camel Milk.'; KO, 'An Overview of the Therapeutic Effects of Camel Milk in the Treatment of Type 1 Diabetes Mellitus'.

The essential ingredients of the camel's milk have been already determined (Haddadin et al., 2008), in that camel's milk is different from other milks by having low sugar, and cholesterol and high concentration of minerals, insulin and vitamins (Korashy et al., 2012a; Korish and Arafah, 2013). Moreover, It has a significant antioxidant effect (Yagil, 2013).

Apart from the significant antioxidant effect(Korish and Arafah, 2013) camels' milk possesses lysozyme, lactoperoxidase and lactoferrin that act as protective proteins (Agrawal et al., 2009; Shamsia, 2009). It has significant effects in lowering microalbuminurea when given as adjunctive therapy in type 1 diabetic patients (Muyldermans, 2001). In addition, camel's milk has been demonstrated, to contain secretory IgA and IgM, numerous non-antibody components that possess antisnake venom inhibitor (Harrison et al., 2003) and antiviral activity (al-Senaidy, 1996). Moreover, alpha-Tocopherol is the predominant form of vitamin E in camel's plasma and tissues, serving as an antioxidant and providing protective mechanisms (Hasson et al., 2004). Until recently, it is traditionally believed that drinking camel's milk has cured some cases of cancer. However, very few studies based on the proliferation and apoptosis associated with human cancer cells have been conducted on the anticancer properties of camel's milk (Bharati et al., 2003; Korish and Arafah, 2013).

Although sufficient number of research has been performed on camel's milk in vitro, the proclaimed health benefits of such anti-cancer candidate have never been adequately investigated. The present study therefore, investigates the efficacy of lyophylised camel's milk to repress the proliferation of human breast cancer BT-474 and HEp-2 cells and explores the underlying molecular mechanisms to initiate its apoptotic- and the oxidative stress-mediated genes to do that the following prospective measured were carried out to evaluate the (i) cell viability (ii) intracellular caspase 3 activation at both the mRNA and activity levels, (iii) evaluation of mRNA expression level of apoptotic caspase-3, p53 and DR4 genes and (iv) intracellular heme oxygenase 1 (HO-1), Caspase-3, DR4, and ROS genes' products as oxidative stress markers.

Materials and Methods

Chemicals

Roswell Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), Trypsin, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). TRIzol reagent was purchased from Invitrogen Co. (Grand Island, NY). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, Trihydrochloride, Trihydrate (life Technologies (H1399), 1,4-diamino-2,3-dicyano-1,4-

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bis(o-aminophenylmercapto) butadiene ethanolate (U0126), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), alpha-Amanitin, 2,7-dichlorofluorescein diacetate (DCF-DA), and 1,9-pyrazoloanthrone (SP600125) were purchased from Sigma Alrdrich Co. (St. Louis, MO). High Capacity cDNA Reverse Transcriptase Kit, and SYBRGreen PCR Master Mix were obtained from Thermo Fisher Scientific (Waltham, MA USA). Caspase-3 colorimetric activity kit was purchased from BioVision (Cat# No. K106-100, Mountain View, CA, USA). Human sense and antisense target primers set sequences obtained from Integrated DNA Technologies, Inc. (Coralville, IA). All other chemicals were acquired from Fisher Scientific Co. (Toronto, ON).

Milk collection and lyophilisation

Milk samples were collected and pooled aseptically from four well-maintained healthy Camelus dromedarius (from His Majesty Sultan Qaboos animals' farm, in Rumais, Oman) and four cows (from local farm in Rumais, Oman), respectively. The milk was collected usually during the feeding time and was performed by expert. Animals were milked into sterile containers directly and transported to the laboratory within two hours. Subsequently, milk samples were centrifuged at 1400°g for 30 minutes at 4°C. Creamy layer consisting largely of fat was separated and discarded by filtration using a glass wool plug in a Pasteur pipette. Free fat milk was collected and lyophilised by freeze-drying, aliquoted and kept at -80°C freezer until use. Working samples, were prepared fresh just before each experiment by dissolving in dimethyl sulfoxide (DMSO) not exceeding 0.05% (v\v). Bovine milk was used as a control and treated in the same way as the camel's milk.

Sterility test

Sterility test was done initially to check the animal products for any contamination. The milks were first filtered through 0.45 μm (Millipore filter) using a 5 ml disposable syringe (Millipore, Bedford, MA) into a sample vial. A 35 mm culture dish was plated with the HEp-2 cell suspension in 2ml of DMEM media and allowed the cells to adhere. The lyophilised camel's milk was added into culture dishes and incubated at 37°C under 5% CO_2 for 24 hours.

Cell line and culture

Human breast cancer cell line (BT-474) Oman [Cell Line Source: the National Cell Bank of Iran (NCBI)]was kindly given by Dr. ShadiaAl-bahlani Department of Pathology, SQU, Oman .The laryngeal (HEp-2) cancer cell line was obtained from the cell bank - Department of Microbiology and Immunology. Whereas the HCC1937 BL, purchased from NGIMS Human Genetics Cell Repository, Coriell Institute of Medical Research (Camden, NJ) (http://ccr.coriell.org/), was used as a negative control (Table 1). BT-474 and HCC1937 BL cell lines were maintained in RPMI-1640 supplemented with 10% FBS, bovine insulin (10μg/ml) 1% penicillin and 1% amphotericin B solution (Fungizone). The HEp-

2 cell line was maintained in RPMI-1640 supplemented with 10% FBS, 1% NEAA and 1% penicillin. All cell lines were grown initially in 75 cm2 tissue culture flasks in a humidified atmosphere under 5% CO_2 at 37°C. Subsequently, cells were seeded onto 24-well cell culture plates according to the need of the assay (see below). Both tested milks were prepared just before each experiment and dissolved in DMSO. All cultures were tested for Mycoplasma contamination and were found to be negative.

Two negative controls were used in the subsequent experiments *(i)* Non-cancereous HCC1937 BL cell line and *(ii)* the use of bovine milk in contrast with untreated cells, neither with lyophilized camel's milk nor with bovine milk.

Effect of lyophilised camel's milk on cell viability

The effect of lyophilised camel's milk on cell viability was determined using the Trypan Blue Exclusion, Hoechst staining and MTT assays as described (Portugal and Waring, 1988; Berridge and Tan, 1993; Strober, 2001), respectively. The MTT assay was used first to determine the optimum milk's concentration to repress the BT-474 and HEp-2 cancer cells' growth and proliferation. The BT-474 and HEp-2 in parallel with control HCC1937 BL cells were treated for 24hrs with increasing concentrations of camel or bovine milks (0, 2.5, 10, 20, 30, 40, 50 and 80mg/mL), thereafter cell viability and proliferation were further confirmed by Trypan Blue Exclusion and Hoechst staining assays

Effect of camel's milk on cell proliferation MTT assay

The MTT assay was performed according to Mosmann (1983) (Mosmann, 1983) with some modifications as described by Denizot and Lanbut (Denizot and Lang, 1986). The efficacy of lyophilised camel's milk to alter BT-474 and HEp-2 cells growth and proliferation in parallel with the healthy cell line were first assessed and determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) salt to formazan crystals as described previously (Mosmann, 1983). After incubating the cells for 24 hours with increasing concentrations (2.5, 10, 20, 30, 50 and 80mg/mL) of the tested milks in a 96-well cell culture plate (5.0 x 104cells per well) [at 37°C under a 5% CO_2 humidified incubator], the media was aspirated and a 100μL of serum-free medium containing 20μL MTT salt (2.5 μg/ml PBS, pH 7.4), was added to each well. The plate was then incubated further in a 5% CO_2 incubator at 37°C for 4hrs. To stop the reaction and dissolve the formed crystals, the media were then discarded by inverting the plate; and a 200μL of 10% sodium dodecyl sulphate (SDS), in deionized water, was added to each well and incubated overnight at 37oC. The color intensity in each well was measured and recorded at 630 nm wavelength, using microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Each determination was carried out in triplicate and two independent experiments were carried out. Cell viability was calculated as percentage and in relation with

the control wells using the formula: Inhibitory rate (%) = (1−A of experiment well/A of control well) × 100%. A dose-survival curve was obtained for each experiment.

In-vitro assay for cell viability (Trypan Blue Exclusion-Assay)

Cell viability assay was carried out by seeding a total of 5.0 x 104 cells\well in 24-well plates. The plate was incubated under 5% CO_2 for 24 hours in a humidified incubator, at 37°C. Then the cells were treated with the test agents and incubated as above for 24, 48 and 72 hrs. Control groups were mixed with bovine milk in DMSO. After 24, 48 and 72 hrs, the cells were harvested by trypsinization and $20\mu L$ of the cell suspension was mixed with $20\mu L$ of trypan blue. The viable cells were counted using a hemocytometer as average number of cell viability (10^4/mL).

Determination of apoptosis using Hoechst staining

Apoptosis was determined further by staining cells with Hoechst stain-33342 and apoptosis percentage was calculated by observing apoptotic features under fluorescence microscope as well as using the formula bellow as previously described by Vashishtha et al., (1998) (Vashishtha et al.).

%Apoptotic cells =

$$\frac{total\ numbr\ of\ cells\ with\ apoptotic\ nuclei}{total\ number\ of\ cells\ counted} \times 100$$

Determination of caspase-3 activity and their cleavage assay

Activity of caspase-3 was measured calorimetrically using the caspase-3 colorimetric assay kit purchased from BioVision (Mountain View, CA, USA) in accordance with the manufacturer's instructions. Concisely, cell lines were seeded onto 12-well cell culture plate and treated for 24 hrs with different concentrations of lyophilised camel's milk as stated before. Thereafter, BT-474 cells were then collected by trypsinization and cell pellets were washed twice with PBS and then resuspended using ice-cold cell lysis buffer and incubated for 10 min on ice. The supernatant was obtained by centrifugation for 10min at 10,000×g at 4°C and transferred to a fresh tube and stored at –20°C until use. To measure and evaluate the caspase-3 activity, about 30µg isolated protein was incubated with 200µM enzyme-specific colorimetric caspase-3 substrate I (Ac-DEVD-pNA), Size: 1 mg, at 37°C for 2hrs. The activity of Caspase-3 was evaluated by measuring absorbance at a wavelength of 505nm, using microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Zymographic assay - catalytic activity

To demonstrate that lyophilised camel's milk exhibits potent and specific pro-apoptotic effects and inhibits the extracellular matrix re-modelling potential of BT-474 cells, catalytic activity was further analysed using zymographic assay.

The gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) conveyed to the conditioned media was detected in gelatinograms as previously described (Martínez-Poveda et al., 2010).

To prepare conditioned media, BT-474 cells were grown in 6-well plates. After 3 days of culture, some wells received 30mg/mL-lyophilised camel's milk. After 24 hrs of incubation, adapted media were harvested and concentrated to 25µg by centrifugation in Amicon Ultra concentration tubes (Millipore). Duplicates were used to determine cell number with a Coulter counter. Aliquots of concentrated adapted media normalized for equivalent cell numbers were subjected to non-reducing SDS/PAGE.

Gelatin zymography gel was performed as previously described by Hasson et al., (2003) (Hasson et al., 2004). Briefly, samples were subjected to non-reducing SDS/PAGE with the addition of gelatin (1 mg/ml) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mMTris/HCl, pH 7.4, supplemented with 2% Triton X-100, and washed twice with 50 mMTris/HCl, pH 7.4. Each wash was with continuous shaking lasted 10 min. After the washes, the gels were incubated overnight at 37°C and immersed in a substrate buffer (50 mMTris/HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mMCaCl2, and 0.02% Na3N). In some experiments, 30mg/mL-lyophilised camel's milk was added to the substrate buffer. Finally, the gels were stained with Commassie blue R-250 and the bands of gelatinase activity could be detected as non-stained bands in a dark, stained background.

Evaluation of the mechanisms associated with apoptosis and oxidative stress

To examine whether the tested milk mediate either apoptosis and/or oxidative stress is cancer-specific, we have to investigate and determine the ability of the lyophilised camel's milk modulate to the expression of apoptotic and oxidative stress genes in human breast cancer BT-474 cells.

Table 1. Cell lines Used in the Study

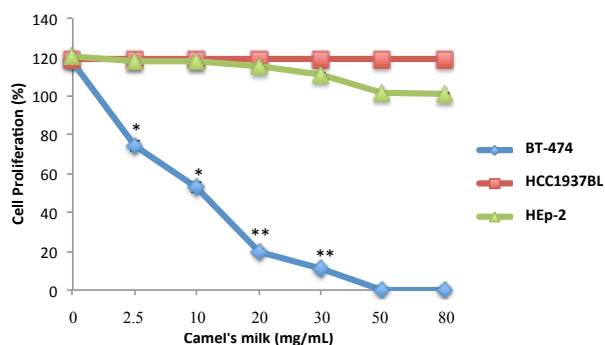| Cell line	Origin	Cell Type	Tissue	Disease	Estrogen Receptor
BT-474	69 years \ Caucasian female	Epithelial	Mammary gland/breast: derived from metastatic site: pleural effusion	Adenocarcinoma	Expressed
HEp-2	56 years\ Caucasian male	Epithelial	Larynx	Carcinoma	Not expressed
HCC1937 BL	24 years Homo sapiens, human female Caucasian	B lymphoblast; Epstein-Barr virus (EBV) transformed	Peripheral blood	Normal	Not expressed

RNA extraction and cDNA synthesis

BT-474 cells tRNA was isolated from 6-well tissue culture plates where the cells had grown to 80-90% confluence. Thereafter, medium was changed to serum free medium and the cells were treated with increasing concentrations (2.5-30mg/mL) of the lyophilised camel's milk dissolved in DMSO for 6 hrs. The total cellular RNA was isolated utilizing TRIzol reagent, as per the producer's guidelines (Invitrogen, Carlsbad, CA), and quantified by measuring the absorbance at 260 nm. The quality of RNA was ascertained by determining the absorbance ratio at 260/280. Subsequently, for reverse transcription-PCR, first-strand cDNA was synthesized from 1μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit with random primers. Concisely, 3 μg of total RNA from each sample was added to a mix of 2.0μL 10X RT buffer, 0.8 μL 25X dNTP mix (100 mM), 2.0μL 10X RT random primers, 1μL MultiScribereverse transcriptase and 4.2μL nuclease-free water. The final reaction mix was kept at 25oC for 10 min, heated to 37°C for 120 min, heated to 85oC for 5 sec, and finally cooled to 4°C.

Real-time PCR analysis of apoptosis-related mRNAs in BT-474 treated cells

Quantitative determination of the expression mRNA was performed by real-time PCR, subjecting the subsequent cDNA to PCR amplification utilizing 96-well optical reaction plates on an ABI 7500 real-time PCR system (Applied Biosystems), using SYBR Green PCR Master Mix. Briefly the reaction mix (25μL) contains 0.1μL of 10μM forward primer and 0.1μL of 10μM reverse primer (40nM final concentration of each primer), 12.5μL of SYBR Green Universal Master Mix, 11μL of nuclease-free water, and 1.25μL of cDNA sample with human forward and reverse target primers as shown below. The PCR primers used to amplify caspase-3, P53, DR4, and heme oxygenase-1(HO-1) genes were 21/18, 17/19, 22/19, and 21/21 nucleotide long oligonucleotides, respectively.

The caspase-3 sequences of sense and antisense strand primers were 5'GAGTGCTCGCAGCTCATACCT and 5'CCTCACGGCCTGGGATTT, respectively. The P53 sequences of sense and antisense strand primers were 5'GCCCCCAGGGAGCACTA and 5' GGGAGAGGAGCTGGTGTTG, respectively. The DR4 sequences of sense and antisense strand primers were 5'AGTACATCTAGGTGCGTTCCTG and 5' GTGCTGTCCCATGGAGGTA, respectively. The heme oxygenase-1(HO-1) sequences of sense and antisense strand primers were 5'ATGGCCTCCCTGTACCACATC and 5'TGTTGCGCTCAATCTCCTCCT, respectively.
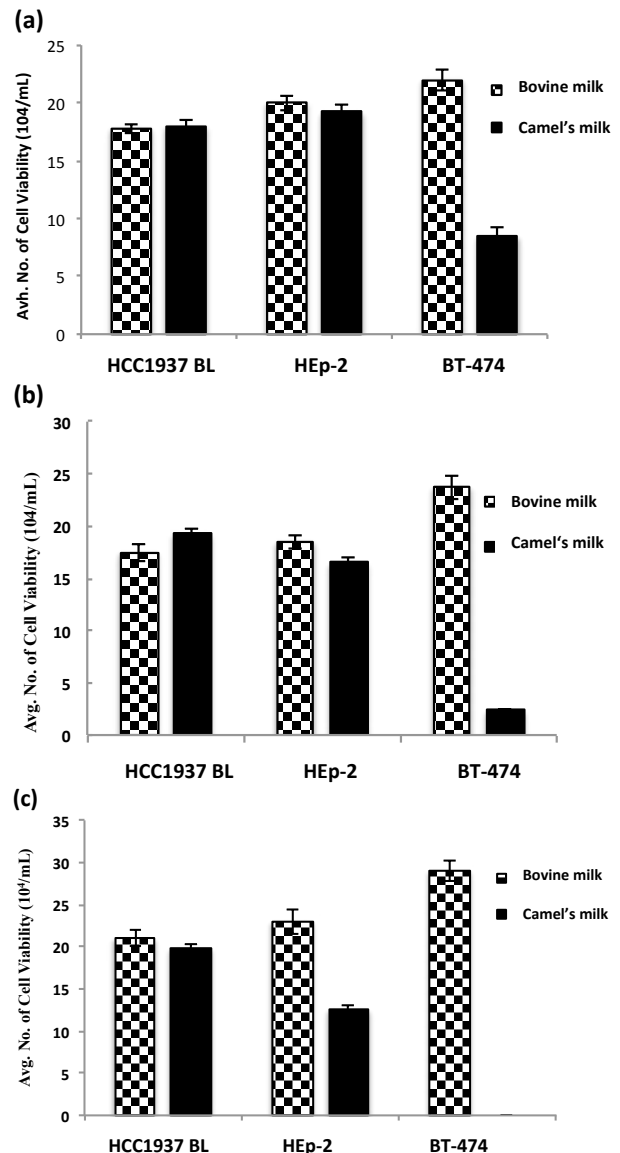
Figure 2. Cytotoxicity effect of lyophilised camel's milk on BT-474 and a time dependent effects on HEp-2 in contrast with bovine milk. Cells were seeded into 24-well plates, with LC_{50} of 9.76 mg/mL was then administered and the number of viable cells was counted after (a) 24hrs, (b) 48hrs and (c) 72hrs and average of viable cells were measured using trypan blue exclusion assay. Each value represents the mean of triplicate determinations. Statistical analysis was determined using Student T test with *p<0.05, ** p<0.003, *** p<0.001, compared to control. Results are shown, as mean±SEM. Data are representative of 3 independent experiments with 3 replicates in each

Figure 1. Effect of camel's milk on BT-474 Cell Proliferation in Contrast with HEp-2 and HCC1937 BL. Cells were seeded and incubated with various concentrations of the tested agent for 24 hrs. Subsequently, cell proliferation was assessed using MTT assay. Values reflected as % in contrast with the number of cells at 0mg/mL concentration (Mean _+SEM, N=2.5). *p<0.006 and**p < 0.001 compared to the control HCC1937 BL. Values at 0mg/ml concentration was taken as 120% cell proliferation. HCC1937 BL is non-cancer cells, refer to Table 1

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The β-actin sequences of sense and antisense strand primers were 5'TATTGGCAACGAGCGGTTCC and 5'GGCATAGAGGTCTTTACGGATGTC, respectively. Untreated samples were merged onto the same plate, marked "controls" to test for the contamination of any reagents used in the assay. Plate was sealed with an optical adhesive cover, qPCR was performed using the following PCR cycling conditions: 95°C for 10 min, followed by 40 cycles of denaturation, each consisting of 95°C for 15s, 60°C for 30s, and 72°C for 30s. Dissociation stage (melting curve) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product. The data are represented as a change in the level of desired gene expression normalized to the housekeeping endogenous gene (β-actin) between the treated and untreated cells and determined utilizing the following formula: fold change old change = $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct(\beta\text{-actin})$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

Effect of the transcription -inhibitor on the induction of caspase-3

To further explore whether the increase in caspase-3 mRNA by the lyophilised camel's milk is attributed to an increase in the newly synthesized RNA, BT-474 cells were treated for 6hrs with the optimum concentration of the lyophilised camel's milk obtained above, the presence or absence of 25μg/mL α-Amanitin, an RNA synthesis inhibitor. Subsequently Caspase-3 mRNA was quantified by RT-PCR. If lyophilised camel's milk influence the increase the amount of caspase-3 mRNA through increasing its new RNA synthesis under these circumstances, we would predict to disclose a diminishing in the content of caspase-3 mRNA after the suppression of its RNA synthesis.

Determination of Reactive Oxygen Species (ROS) production

Production of intracellular ROS was determined and analyzed fluorometrically by measuring the oxidation of a non-fluorescent probe 2,7- dichlorofluoresceindiacetate (DCFH-DA) to a fluorescent metabolite dichlorofluorescein (DCF) by the mitochondrial ROS as described previously with slight modifications (Bland et al., 2001; Rastogi et al., 2010). Concisely, cells allowed to reach a growth of 90% confluence in 96-well cell culture plates and treated for 24 hrs with different concentrations of lyophilised camel's milk. Subsequently, cells were washed with PBS before incubation for 30 min in new prepared media containing 10μM DCF-DA. The fluorescence was directly measured using excitation and emission filters of 485 and 535 nm, respectively, with a POLARstar Optima plate reader (BMG Labtech, Offenburg, Germany)

Statistical analysis

Data are presented as mean +/- ± SEM of two independent experiments. Statistical significance was determined using independent sample t-test, SPSS software (SPSS v.20). The differences were considered significant when P values are<0.05.

Results

MTT assay

MTT assay have shown anticancer activity of the lyophilised camel's milk through significant cytotoxic activity against human BT-474 cell line with LC50 of 9.76 mg/mL (Figure.1).

As the control HCC1937 BL cells showed high proliferation which has been taken as 120% of viable cells regardless of the camel's milk concentration, cell viability of the BT-474 was found to be suppressed significantly over 95% at a concentration 47mg/mLp<0.006 (Figure 1) when compared to those of the HEp-2 and HCC1937 BL and in comparison with those of untreated cells (data not shown) that showed high proliferation. Hence the activity was more selective to BT-474 cell when compared with HEp-2 and normal cell (HCC1937 BL) with spatial-selectivity indices >0 indicating higher activity.

Trypan blue exclusion assay

The trypan blue test results for 24, 48 and 72 hrs incubation of lyophilised camel's milk with the cell lines showed cytotoxic activity with the LC50concentration on the growth of BT-474 and HEp-2 human cancer cell lines in contrast with the normal cell line and the use of bovine milk. Lyophilised camel's milk actively exhibited significant cytotoxic effect immediately after 24 hrs Figure 2a (p < 0.05) and Figure 2b (p<0.003). Figure 2a reveals a prompt cytotoxic effect 24hrs after the introduction of the lyophilized camel's milk on the BT-474 in contrast with both the HEp-2 and the normal cells. Interestingly, although the lyophilised camel's milk showed a significant toxicity against the BT-474 after 48 and 72hrs it also showed some effect on the HEp-2 cells viability in a time dependent manner Figure 2b and Figure 2c (p < 0.003) in contrast with its results at 24 and 48hrs with the normal cell control.

Hochst 33342 DNA Staining

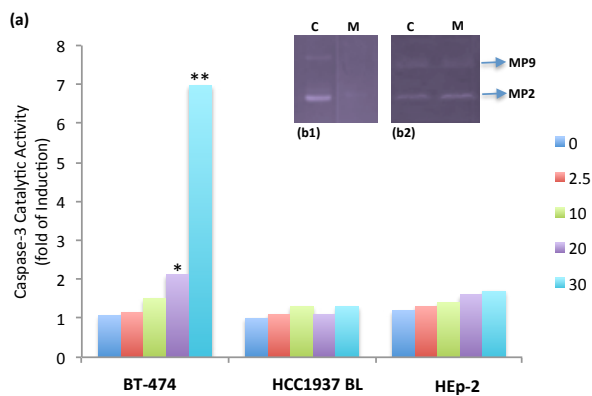The inhibitory effect of the lyophilised camel's milk
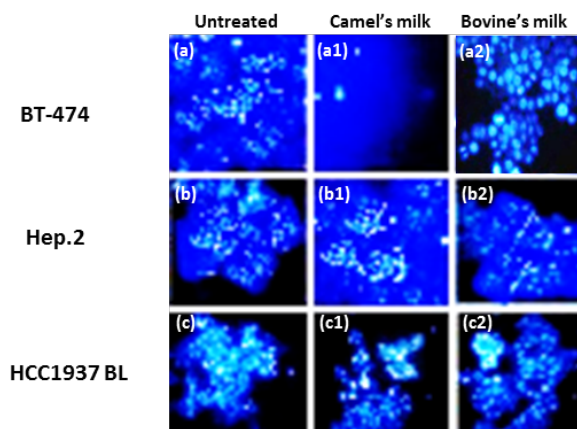
Figure 3. Apoptosis of (a) BT-474 and (b) HEp-2 breast cancer cell lines as observed under fluorescence microscope after 24 hrs of adding lyophilised camel's milk. Healthy cell line (c) HCC1937BL illustrated in parallel. All cell lines shown were treated with lyophilised camel's and bovine's milks as indicated.

on BT-474 and HEp-2 cells was further confirmed using Hoechst 3342 staining assay. The assay have shown complete lysis of BT-474 and HEp-2 cells after treating them with lyophilized camel's milk in contrast with that of the HEp-2 and HCC1937 BL (Figure.3b1 and c1) as it showed partial cell lysis. Apoptosis of BT-474 cells by lyophilized camel's milk were characterized by complete lysis and or cytoplasmic shrinkage.

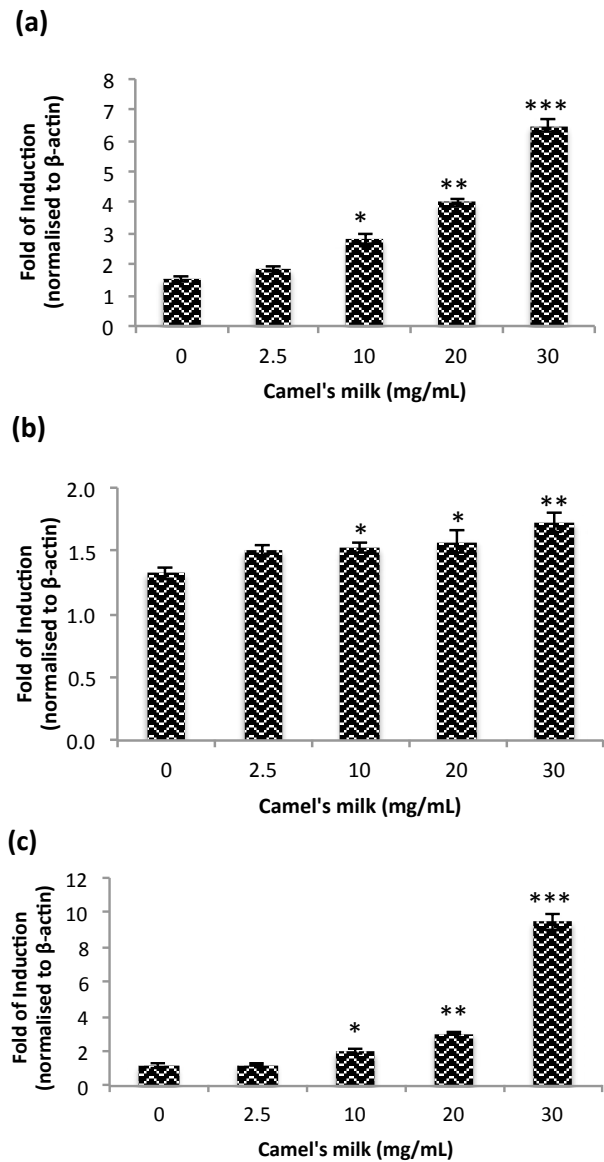*Evaluation of intracellular caspase 3 activity following treatment with the lyophilized camel's milk*

Intracellular Caspase 3activation is a key stage in the apoptotic pathway. Hence, we explore the efficacy of the lyophilized camel's milk on caspase-3 activity. For this purpose, BT-474 cells were treated for 48hrs with increasing concentrations of camel's milk; subsequently, caspse-3 activity was evaluated colormetrically using BioVision kit. In this experiment, total caspase-3 activity from the entire cell population was measured. Since the activity of caspase-3 ascents while cells die and cell numbers drop, it was essential to normalize caspase-3 activity to the number of cells, to obtain more accurate results. Therefore, identical samples were analyzed simultaneously for cell viability. The results here are expressed as the fold increases in caspase-3 activities in treated cells compared to normal cells at the same final concentration as in the control cells. Figure 4 shows that lyophilised camel's milk increased the caspase-3 activity only at the higher concentrations, 20 and 30 mg/mL by approximately 3.2 and 6.9 folds after 48 hrs, respectively, suggesting an induction of apoptosis.

*Zymographic assay - catalytic activity*

To test the effects of lyophilised camel's milk pre-treatment on BT-474 extracellular matrix degrading enzymes were analysed. Our results with gelatin zymography showed that 30mg/mL lyophilised camel's milk completely inhibits MMP-2 secretion by the BT-474 cells, but does not inhibit its activity directly when added to zymography substrate buffer (Figure 4b1and b2).

(a)

(b)

(c)

Figure 4. Influence of Lyophilised Camel's Milk on the "apoptotic" caspase-3 catalytic activity in BT-474 Cells. (a)Cells were treated with various concentrations of camel's milk (2.5, 10, 10, 20 and 30 mg/mL) and incubated for 24hrs. Subsequently, evaluation of caspase-3 activity was assessed calorimetrically using the CaspACE assay (BioVision). Caspase- 3 activity was determined by recording the absorbance at a wave length of 405 nm. Values represented here as mean ± SEM (n = 5). P values were calculated against the control (0 mg/ml). *P0.01, **P<0.01. (b)For zymographic assays, conditioned media (for gelatinolytic assays) was normalized for equal cellular density. (b1) shows camel's milk added to zymography substrate buffer. (c1) conditioned media of BT-474 cell line. C=control; M= lyophilised camel's milk [30mg/mL]

Figure 5. Effect of lyophilised camel's milk on apoptotic markers. (a) Caspase-3; *P<0.05, **p<0.004 and p<0.0001, (b) p53; *p< 0.05 and ** p<0.02 and (c) DR4; *<0.01, p<**0.001 and ***p<0.0001, mRNA levels in BT-474 cells. BT-474 cells were treated with various concentrations of lyophilised camel's milk (2.5, 10, 20, and 30 mg/mL) and lasted for 4 hrs. Subsequently, the total RNA was isolated using TRIzol reagent, and the mRNA levels of each caspase-3, p53 and DR4 genes were quantified using RT-PCR normalized to β-actin housekeeping gene (see text). Triplicate reactions were performed for each experiment, and the values presented as the means ± SEM (n =5) of each of the three independent experiments. P values were calculated against the control (0 mg/ml)
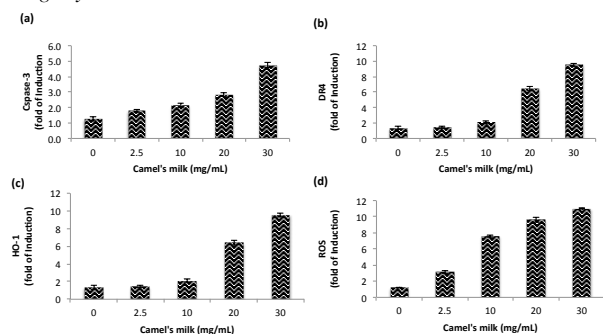
Figure 6. Effect of Lyophilised Camel's Milk on Apoptotic and Oxidative Stress Markers. (a) Caspase-3, (b) DR4, (c) HO-1 mRNA levels and (d) ROS production in BT-474 cells. BT-474 cells were treated with various concentrations of camel's milk (2.5, 5, 10, 20 and 30 mg/mL) for 4hrs. Subsequently, the total RNA was isolated using TRIzol reagent. RT-PCR normalized to β-actin housekeeping gene was used to quantify the levels of the mRNA of caspase-3, DR4, and HO-1 genes. DCF formation was measured fluorometrically using excitation/emission wave lengths of 484 and 535nm, respectively. Triplicate reactions were performed for each experiment, and the values presented as the means ± SEM (n=5) of each of the three independent experiments. $P<0.05$ values were calculated against the control (0 mg/ml)
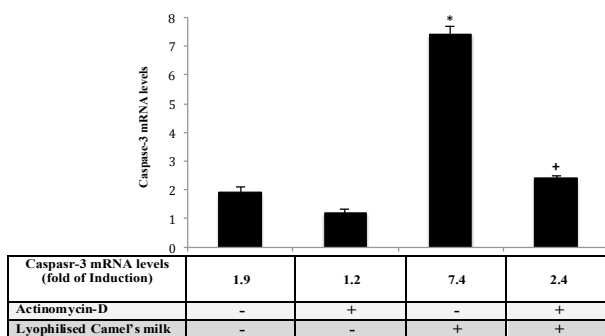
Figure 7. Effect of Act-D on the caspase-3 Activity by the Lyophilised Camel's Milk in BT-474 Cells. The BT-474 cells were treated with 25 μg/mL RNA union inhibitor "Act-D", 20 min prior introduction to the tested agent (30 mg/mL) for 4 hrs. The caspase-3 mRNA was evaluated quantitatively utilizing RT-PCR and normalized to β-actin housekeeping gene. Triplicate reactions were performed for each experiment, and the values presented are the means ± SEM (n = 5) of three independent experiments. Triplicate reactions were performed for each experiment. The values represented here are the means ± SEM (n = 5) of three independent experiments. *$P<0.05$ compared with control; +$P < 0.05$ compared to same treatment in the absence of Act-D

Evaluation of mRNA expression level of apoptotic genes in BT-474 following treatment with the lyophilized camel's milk

To assess whether the inhibitory effect of lyophilized camel's milk on BT-474cells proliferation and growth is an apoptotic-mediated mechanism, we evaluated the capability of lyophilized camel's milk to initiate the expression of apoptotic and anti apoptotic genes. For this intent, BT-474 cells were incubated for 6 hrs with increasing concentrations of lyophilized camel's milk

(2.5, 10, 20, and 30mg/mL), as determined previously by the MTT assay (Figure 1). Hence, caspase-3, p53 and DR4 mRNA expression levels were then established by RT-PCR, the results shown in Figure 5 illustrate that the lyophilized camel's milk significantly instigated caspase-3 and p53 mRNA expression levels in a concentration-dependent manner (Figure 5a and 5b), respectively. The maximum induction was observed at the highest concentrations tested (30 mg/mL) by approximately 6.5 and 8.5 folds, respectively. Then again, slight changes in DR4 mRNA levels were found and directly proportional to the increase in concentration of the lyophilized camel's milk as illustrated in (Figure 5c).

Influence of lyophilized camel's milk in Initiation of the expression of oxidative stress genes in BT-474 Cells.

To further study whether the apoptotic genes that incited by the tested agent is correlated with inducement of oxidative stress markers in BT-474 cells, we evaluated the potential of tested agent on hemeoxygenase 1 (HO-1), caspase-3, DR4, and ROS production as oxidative stress markers. For this purpose, BT-474 cells were incubated in different concentrations of the lyophilised camel's milk for 6hrs. Subsequently RT-PCR and DCF assay were used to determine caspase-3, HO-1 and DR4 mRNA levels (the markers that showed significant alterations in BT-474 cells) as well as ROS production. Figure 6b and 6c illustrate that the lyophilised camel's milk significantly influence the induction of DR4 and HO-1 mRNA levels only at the higher concentrations tested, i.e., 20 and 30 mg/mL, by almost 3 and 4 folds, respectively. In addition, results of the caspase 3 were gradually increased in a concentration-dependent manner (Figure 6a). Moreover, Figure 6d shows that ROS production significantly increased at all concentrations tested with a significant low production at a concentration of 2.5 mg/mL.

Effect of the transcription inhibitor on the induction of caspase-3

Figure 7 demonstrate that pretreatment of the cells with Act-D did not fundamentally modify the constitutive expression of caspase-3 mRNA when contrasted with untreated cells. On the other hand, the provoking of caspase-3 mRNA by lyophilised camel's milk was totally nullified by Act-D, proposing that lyophilised camel's milk builds the caspase-3 mRNA level by expanding its new RNA synthesis.

Discussion

This study demonstrates that lyophilized camel's milk, but not bovine milk, was able to significantly suppress BT-474 cells proliferation and growth in comparison with HEp-2 and HCC1937 BL cell lines (Figure 1). In spite of the fact that the dynamic components of camel's milk involved in apoptosis were not investigated in this study, a few past studies have found that camel's milk contains significantly high amounts of antioxidant vitamins, such as E and C (Farah et al., 1992), lyzosomes (Agamy et al., 1992), lactoferrins (Agamy et al., 1992) and immunoglobulins (Konuspayeva et al., 2007).

Lactoferrin, an iron-binding glycoprotein, is known to have antitumor activity (Roseanu et al., 2010). Therefore, it may be accountable for the cytotoxicity effect exerted by lyophilized camel's milk. The anticancer activity of the camel's milk lactoferrin against HCT-116 colon cancer cells was proven in a study performed by Habib et al. (2013) (Habib et al., 2013). However, no studies have been conducted so far to demonstrate its antitumor activity against BT-474 and HEp-2 cancer in parallel with the non-cancerous cell line.

The current study shows that lyophilized camel's milk caused significant cytotoxicity effect against the BT-474 cell line after 24hrs (Figure 2c). Such toxicity also was found to be against the HEp-2 cell line but at 72hrs (p< 0.05). This may be explained by estrogen receptor expression differences between the two cell lines. Since estrogen receptor is expressed by BT-474 cell line but not by HEp-2.This may speculate that the main target for the lyophilized camel's milk to initiate apoptosis in BT-474 cell line is the estrogen receptor whereas an alternative non-estrogen receptors path way may have mediated apoptosis in HEp 2 cell line. This was confirmed further by the low in cell density observed using the Hochst 33342 DNA Staining assay (Figure 3c1).

The activation of caspase-3 plays a major role in the initiation of apoptosis, which requires the activation of initiator caspases, such as caspase-8 or -9, in response to proapoptotic signals (Lowe, 2000). The current study demonstrates that the lyophilised camel's milk-mediated effect in BT-474 cells is attributed to induction of apoptotic signaling pathway. This was supported further by activation of caspase-3 mRNA and its activity levels with no changes in the expression p53 (Figure 5b), suggesting a p53-independet mechanism. Although p53gene was the first tumor suppressor gene linked to apoptosis, in 2001 when Matusi and his team demonstrated that, DNA-induced apoptosis found to be triggered in cell lines lacking p53 expression or mutated p53 (Matsui et al., 2001). Other studies have reported the ability of oleuropein, a type of phenolic compound found in olive leaf (Hassan et al., 2014b), and thymoquinone extracted from black seeds (Gali-Muhtasib et al., 2008), Paeonia Suffruticosa Andrews (Choi et al., 2012) to activate apoptosis through a p53-free pathway utilizing transgenic and knockout mice.

Apoptosis can be induced through the activation of death receptors including the cell-surface death receptors such as Fas receptor (Fas), also termed Apo-1 or CD95. These receptors have been identified as major apoptotic inducers (Johnston et al., 2003; Elmore, 2007). A cell that dies after initiation of the Fas receptors by caspases activation normally shows an apoptotic cell death. Enhanced Fas expression in cells instigated by cytotoxic drugs, especially those that are known to result in DNA damage and are presently in remedial utilization, suggests a combined approach to chemotherapy using drug/anti-Fas mAb combinations. Therefore, we have examined whether DRs are included in camel's milk-interceded impacts by measuring the declaration of DR4 (Figure 5c) and found that induction of DR4 mRNA in light of camel milk fundamentally contributes to the initiation of caspase-3

and its mechanism. Our results are in concurrence with previous findings, which demonstrated that antitumor agents, for example, ET, doxorubicin, Ara- C, 5-FU, and cis-platinum, or irradiation could up direct the expressions of both DR4 and DR5 in multiple myeloma, acute leukemia, and solid tumor cell lines [65]. Furthermore, impelling of apoptosis with generation of ROS by cancer chemo protective agents, such as doxorubicin or any other agent(Tsang et al., 2003), not only induces cancer cell death but also causes DNA damage and genomic instability (Zhivotovsky and Kroemer, 2004). In spite of this the use of cytotoxic chemotherapy is fraught with toxicity (Feng et al., 2014). Hence, one of the strategies for cancer development suppression includes attenuation of anti-apoptotic genes. Therefore, the development of new chemopreventive agents, which are able to repress cell proliferation and induce apoptosis in cancer cells, is a plausible option to explore. For these reasons, we evaluate further the capacity of the lyophilised camel's milk to invigorate pre-apoptotic signals, and induction of ROS production.

Lyophilised camel's milk apoptotic markers activation of caspase-3 has also shown to be mediated through mitochondrial ROS production (Ravagnan et al., 2002). This data provide further evidence that the apoptotic cell death induced by the lyophilized camel's milk in BT-474 cell line might be associated with the generation of ROS. This is in concurrence with the previous study that reported ROS per se as potent inducer of apoptosis obtained (Kroemer, 1997). Taken this together, it seems that induction of ROS by the lyophilized camel's milk may have a direct role in the increased level of the expressed mRNA of ROS (Figure 6). This provides further evidence that the apoptotic cell death induced by lyophilised camel's milk in BT-474 cells is associated with ROS generation. This is in agreement with previous findings that ROS essentially are potent inducers of apoptosis (Ketola et al., 2010). Interestingly, comparative example of caspase-3, DR4, and HO-1 mRNAs induction was seen in Larynx HEp-2 cells cancer (Data not shown), suggesting that camel's milk mediated effect is not specific to HEp-2 cells. Taken together, these results indicate that BT-474 cells are prone to caspase-3-induced cell death, as overexpression of caspase-3 induced direct cytotoxicity to the cells but with no effects has been observed to the control cell line (data not shown).

Confirmation of the catalytic activity of the caspase 3, illustrated in Figure 4a, was further assessed by zymography assay (Figure 4b) which showed that the induction of BT-474 cell apoptosis down-regulates the expression/release of matrix metalloproteinases, thus reducing its invasive potential. This is in agreement with the previous observation by Zhivotovsky and Kroemer (2004)(Zhivotovsky and Kroemer, 2004). These finding point to a certain specificity of the effects of lyophilised camel's milk for BT-474 cells.

Results obtained by the addition of the transcription inhibitor, Act-D, (Figure 7) were found to be significant and supported the ability of the lyophilised camel's milk to initiate the transcriptional related mechanism of the target caspase-3 gene. Suggesting a prerequisite of newly

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synthesized RNA for the inducement of caspase-3 mRNA by the lyophilised camel's milk.

Lyophilised camel's milk exhibits potent and specific pro-apoptotic effects and inhibits the extracellular matrix re-modeling potential of BT-474 cell. Consequently, through its apoptotic and oxidative- stress-mediated mechanisms properties, the lyophilised camel's milk can be regarded as a novel candidate drug of great interest for the development of new therapeutic strategies for treatment of breast cancer.

Acknowledgements

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