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Full Length Research Paper

Effects of *Tinospora crispa* aqueous extract in regulating cholesterol metabolism in human hepatoma cancer cell line (Hep G2)

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In this study, the ability of *Tinospora crispa* aqueous extract (TCAE) to regulate cholesterol metabolism in human hepatoma cancer cell line (Hep G2) was determined. Cytotoxic study was performed by exposing hepatoma cell (Hep G2) towards TCAE with concentration ranging from 0.002 to 20 mg/ml for 24 h at 37°C and with 5% CO_2 atmosphere. Result revealed that TCAE was not toxic to the cell. The ability of TCAE to reduce cholesterol in cell culture experiment was carried out by pre-treating Hep G2 with selected concentrations of TCAE (10, 5, 2.5, 1.25 and 0.625 mg/ml) in 6-well plate before the cell was exposed to low density lipoprotein (LDL). The concentration of apolipoprotein A1 (Apo A1), lecithin-cholesterol acyltransferase (LCAT), low density lipoprotein receptor (LDLR), scavenger receptor B1 (SRB1) and hepatic Lipase (HL) which involve in reverse cholesterol transport (RCT) pathway were determined from the 6-well plate medium. The direct pathway of cholesterol synthesis was performed according to the instruction provided in HMG-CoA Reductase Assay Kit manuals. The results showed that TCAE significantly increase ($p<0.05$) the concentration of Apo A1, LCAT, LDLR, SRB-1 and HL. The efficacy of these activities is appreciably good when compared with standard drug simvastatin. However, TCAE showed moderate effect in controlling mevalonate pathway. These findings suggested that TCAE has the potential to reduce cholesterol metabolism in Hep G2 cancer cell lines and the pathway of TCAE action possibly more on RCT.

Key words: *Tinospora crispa*, cholesterol metabolism, reverse cholesterol transport, cytotoxic, Hep G2.

INTRODUCTION

Liver is a very important organ which plays a pivotal role in cholesterol metabolism. Cholesterol is synthesis in hepatocytes through mevalonate pathway with the help of HMG CoA Reductase (HMGR) (Petras et al., 1999) and secreted into bloodstream as a forward pathway in order to supply cholesterol to peripheral cells (Friedman et al., 2009). Conversely, reverse cholesterol transport (RCT), the process by which excess cholesterol is effluxed from

cells into high density lipoprotein (HDL) particles, helps to carry out excess cholesterol and returned to the liver for excretion from the body (Ghosh, 2010; van der Velde et al., 2010). This process is crucial in regulating cholesterol homeostasis by facilitating the prevention of lipid accumulation, particularly, in atherosclerotic lesions and therefore, RCT is considered an atheroprotective process.

RCT is a complex process that encompasses some protein molecules and enzymes such as apolipoprotein A1 (Apo A1) (Getz and Reardon, 2011), lecithin-cholesterol acyltransferase (LCAT) (Zannis et al., 2006), scavenger receptor B1 (SRB1) (Zannis et al., 2006), hepatic Lipase (HL) (Brown et al., 2010) and low density lipoprotein receptor (LDLR) (Carneiro et al., 2012). Stimulation of reverse transport of cholesterol from cells of the arterial wall to the liver is considered the mechanism by which HDL exerts its anti-atherogenic properties. In addition, interaction of HDL with the arterial wall directly protects against oxidative stress and vascular inflammation (van der Velde et al., 2010). Therefore, low level of HDL is considered as an important risk factor for the development of atherosclerosis (Ghosh, 2010).

Tinospora crispa aqueous extract (TCAE) cholesterol reducing abilities has been explored earlier and it is proven that TCAE can reduce the plasma low density lipoprotein (LDL), total cholesterol (TC), and malondialdehyde (MDA) level on hypercholesterolemic-induced models. It was reported that, TCAE exerted significant results in decreasing the cholesterol level similar to what statin does (Zulkhairi et al., 2009). Despite the promising evidences of its ability in lipid lowering activity, information on the underlying mechanism of action which contributes to the effect is not well documented so far. Thus, it is important to investigate the effects of this plant in regulating the cholesterol metabolism through their essential biocomponents involved in either forward or reverse cholesterol transport pathways including HMGR, SRB1, Apo A1, LDLR, HL, ACAT, and LCAT in order to determine its specific mechanism of action. Therefore, the aim of this study is to investigate the TCAE effects in regulating cholesterol metabolism in Hep G2 cells.

MATERIALS AND METHODS

Cell lines and chemicals

Experimental Hep G2 cell line was purchased from ATCC (American Type's Tissue Culture, USA) and maintained as recommended. Simvastatin, penicillin/streptomycin, trypane blue, fetal bovine serum (FBS), dimetyl sulfoxide (DMSO), 3-4,5 dimetyl tiazol-2, 5 difenyl tetrazolium bromide (MTT) and phosphate buffer saline (PBS) were purchased from Sigma, USA. Instruments used in this experiment were CO_2 incubator (Shelab, German), hemocitometer (La Fontaine, Perancis), vacuum pump, multiple pipet (RAININ, USA), homogenizer (Hettich, Zentrifugen, German), water bath (Jeiotech, Korea), microplate reader (UVM 340, German), reverse microscope (Nikon Gerhana TS100) and micro centrifuge.

Preparation of plant extract

Preparation of TCAE was done according to the method by Kamarazaman et al. (2012) with slight modification. About 10 kg of fresh stem part of *T. crispa* were collected from Forest Research Institute Malaysia (FRIM) at Kepong, Selangor. The plant was authenticated by FRIM botanist (Voucher number: SBID009/15). The stems were cleaned, washed, cut and dried using an oven dryer with operating temperatures of about 55°C. The weight of the samples was monitored every day until constant weight was obtained. Subsequently, the dried stems of the plant are ground to a particle size of about 1 to about 4 mm by using a 20 hp pilot scale grinder. Then about 100 g dried *T. crispa* was soaked in 900 ml of distilled water ratio for 24 h at room temperature. The mixture was incubated in the shaking water bath at 60°C for 6 h. The mixture was filtered and freeze-dried. The crude extract of TCAE was kept at -20°C until use.

Cell culture and maintenance

Hep G2 was cultured in RPMI 1640 media, supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. Hep G2 cell was cultured on 75 cm^2 flask in a humidified atmosphere containing 5% CO_2 incubator at 37°C. The cells were grown to confluences before treatment of TCAE.

Cytotoxicity screening: Determination of TCAE IC_{50}

Cytotoxic assessment of TCAE were performed according to the method by Ibrahim et al. (2010) by exposing the Hep G2 cell G2 (ATCC, HB8065TM, USA) to TCAE (ranging in concentration from 0.002 to 20 mg/ml) for 24 h at 37°C and 5% CO_2 atmosphere, in order to determine the inhibition concentration 50 (IC_{50}) of TCAE; concentration that killed 50% of cell population in well plate, that can jeopardize the cholesterol metabolism due to the cell death. After 24 h of incubation with the extract, 20 µl of MTT (final concentration 0.5 mg/ml) solution was added into the 96-well plate and the plate was further incubated into a CO_2 incubator at 37°C for 4 h. After that, the media was discarded and 100 µl of DMSO was added to each well to dissolve formazan crystals. The plate was read at 570 nm by using microplate reader. The experiment was done in triplicate. The percentage of cells viability was calculated as:

Percent of viability (%) = Absorbance of the treated cell / Absorbance of control cell

Determination of TCAE HMGR inhibition activity using enzyme assay kit

In this study, the ability of TCAE to reduce cholesterol synthesis

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was performed according to the instruction provided in HMG-CoA Reductase Assay Kit manuals (Catalog Number CS1090) obtained from Sigma Aldrich. *T. crispa* extract (5 and 10 mg/ml) was freshly prepared. The TCAE samples or simvastatin was added with nicotinamide adenine dinucleotide phosphate (NADPH) and HMG-CoA substrate solution in 1 ml cuvette. Blank was added with the same substrate and enzymes solution, without sample or simvastatin. The reaction was started by adding HMG-CoA reductase (HMGR) to all cuvettes. The absorbance reading was performed kinetically at every 1 min for 10 min by using UV spectrophotometer at 340 nm wavelength. The experiment was done in triplicate. The calculation of HMGR activity was conducted according to the equation:

$$\text{Units/mgP} = \Delta A_{340}/\text{min}_{\text{control}} - \Delta A_{340}/\text{min}_{\text{sample}}$$

Determination of Apo-A1, SRB1, LDLR, HL and LCAT using cell culture

In this study, the ability of TCAE to reduce cholesterol metabolism in human hepatoma cancer cell line (Hep G2) was carried out following the method by Hubert et al. (2001) and Peter et al. (2010) with slight modification. This experiment was carried out after the effective concentration of TCAE had been identified. Five TCAE concentrations (0.625, 1.25, 2.5, 5, and 10 mg/ml) were selected according to cytotoxicity assay which showed that it was not toxic to the Hep G2 cell. The cell was plated in 6-well plate at the concentration of 1×10^6 cells/well followed by incubation in CO_2 incubator at 37°C for 48 h. The cells were divided into 4 groups: normal control (N) group (Hep G + medium only), untreated (NC) control group (Hep G2+ 10 µl LDL), pravastatin control group (Hep G2 + 10 µl LDL + 10 µM simvasttatin) and treatment groups (Hep G2 + 10 µl LDL + TCAE (10, 5, 2.5, 1.25 and 0.625 mg/ml). The plate was incubated in a 5% of CO_2 atmosphere at 37°C for 24 h. After 24 h of incubation, the media was collected into falcon tube and centrifuged at 1000 rpm. The supernatants were collected for the determination of Apo A1 (AssayMax, Catalog No: EA5301-1), LDLR (Wuhan EIAAB science Co., LTD, Catalog No: E91008Hu), LCAT (Wuhan EIAAB science Co., LTD, Catalog No: E98516 Hu), SRB-1 (Wuhan EIAAB science Co., LTD, Catalog No: E1530Hu) and HL (Wuhan EIAAB science Co., LTD, Catalog No: E0769Hu). The procedures of the experiments were according to the instruction provided in the manual kit. The experiment was done in triplicate.

Statistical analysis

All data were analyzed using the computer software Statistical Package for Social Sciences (SPSS) version 20.0 and were expressed as mean + standard deviation. Comparisons of group means were done by one-way analysis of variance (ANOVA) with a probability less than 0.05 ($p < 0.05$) taken as indicative of significant difference. The mean value (x) and standard deviation (SD) were calculated for each variable measured. Turkey's pos hoc test was used for multiple group comparison. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Cytotoxicity screening of TCAE

In vitro cytoxicity screening in the present study was exercised to determine the optimum concentration of TCAE, since beside their therapeutic potentials TCAE, may also cause adverse effect. This is to ensure that the concentrations selected will not cause necrosis to the cell which later may disrupt cholesterol metabolism activity of Hep G2; hence, the effects of both TCAE in regulating cholesterol metabolism cannot be monitored.

The uses of Hep G2 in this study is due to its ability to retain normal cholesterol metabolism, that is, normal hepatocytes cell (Hasan et al., 2015; Dashti, 1992; Yanagita et al., 1994) and its ability to express several genes involved in cholesterol homeostasis including LDLR, HMGR, Apo A1, LCAT, and SRB1. Hep G2 cell line was reported to synthesize cellular triglycerides and cholesterol and has been widely used on cholesterol synthesis and metabolism study (Funatsu et al., 2001; Scharnagl et al., 2001).

Result from the present study revealed that, the treatment of TCAE from concentration ranging from 0.002 to 20 mg/ml did not cause 50% cell death to Hep G2 cell population, indicating that the concentrations used did not impede cholesterol metabolism activity of the cell. This data is in accordance with Zulkhairi et al. (2008) who reported *T. crispa* extract with concentrations varying from 50 to 900 µg/ml showed no toxic effect on HUVEC cell, normal cell lines ($p<0.05$) (Figure 1).

It is also found that *T. crispa* extract produced no toxic effect on brine shrimp survival and does not demonstrate any IC_{50} even up to an extreme concentration of 1 g/ml. Similar finding was reported by Tungpradit et al. (2010) who stated that *T. crispa and Tinospora cordifolia* water and methanol extracts had no significant cytotoxicity to HL 60, Hep G2 and MCF-7 cancer cells with the IC_{50} up to 500 µg/ml. Moreover, many previous studies done on *T. crispa* in several experimental animals had reported no evidence of organ damage (Pingale, 2011; Talubmook and Buddhakala, 2013; Abu et al., 2015)

However, our finding was contradicted with Md et al. (2011) who found that chloroform, petroleum ether and methanol extract of *T. cripsa* exhibited very significant cytotoxicity with IC_{50} value of 11.5, 12.6 and 12.0, µg/ml, respectively, in the brine shrimp lethality bioassay. The reason for the differences could be due to different extraction medium used in which most of those chemicals although reported to be useful but they are also very toxic (Pruthu, 2015). The use of water also is in line with recent trends in extraction techniques known as green extraction which largely focused on finding solutions that minimize the use of solvents, while also enabling process of strengthening and a cost-effective production of high quality extracts (Chemat et al., 2012). Meanwhile, it was reported that 10 µM simvastatin caused no cytotoxic effect in the HepG2 cells (Peter et al., 2010).

Effects of TCAE in regulating cholesterol metabolism in HEP G2 cells

Reverse cholesterol transport (RCT) is a pathway by

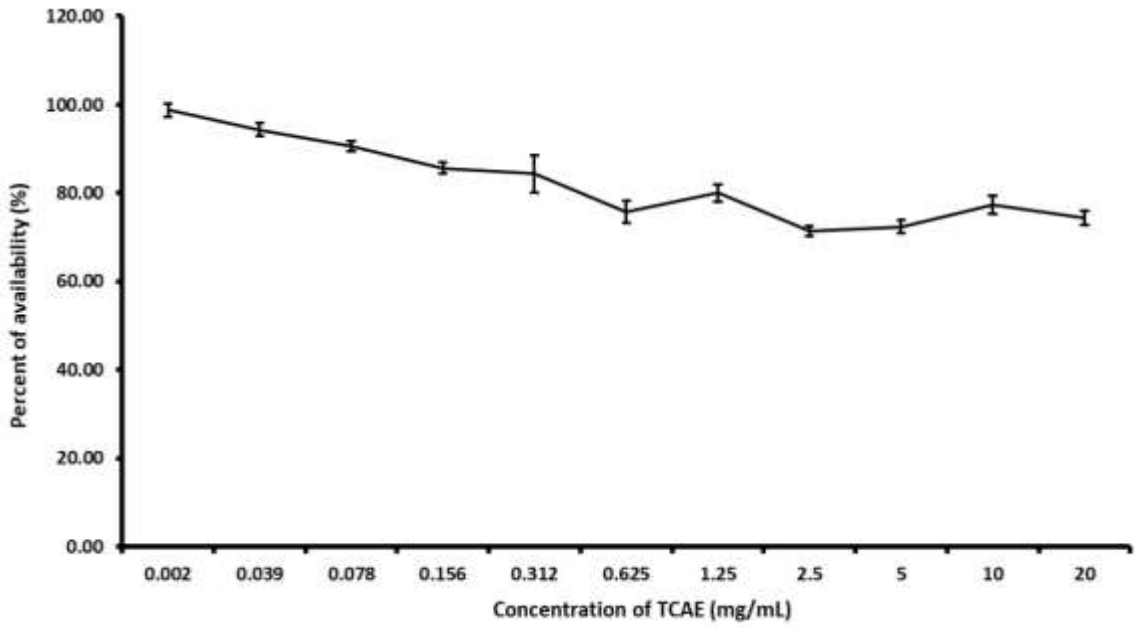

Figure 1. Percentage of viability of Hep G2 cells in 96-well plate against concentration of TCAE. The cells were seeded in a 96-well plate in amount of 1×10^4 cell per well and were nurtured with 100 µl media (RPMI 1640) per well. The cells were treated with different concentration of TCAE and incubated in 5% CO_2 humidified incubator at 37°C for 24 h.

which accumulated cholesterol is transported from the vessel wall to the liver for excretion, thus preventing atherosclerosis. Major constituents of RCT include acceptors such as HDL and Apo A-I, and enzymes such as LCAT, hepatic lipase (HL) and cholesterol ester transfer protein (CETP). A critical part of RCT is cholesterol efflux, in which accumulated cholesterol is removed from macrophages in the sub intima of the vessel wall by ATP-binding membrane cassette transporter A1 (ABCA1) or by other mechanisms, including passive diffusion, scavenger receptor B1 (SR-B1), and collected by HDL and Apo A-I. RCT plays a major role in anti-atherogenesis and modification of these processes may provide new therapeutic approaches to cardiovascular disease (Ohashi et al., 2005).

Apo A1

The results of the Apo A1 are shown in Figure 2. Result showed that TCAE was able to stimulate Hep G2 cell to secrete APO A-1 although it is not comparable to that of simvastatin. The Apo A1 concentration of groups treated with 10, 5, 2.5, 1.25 and 0.625 mg/ml of TCAE were increased by 52.11, 50.72, 37.82, 39.96, and 26.28%, respectively, whereas simvastatin increased Apo A1 levels by up to 101.82%, compared to NC group. Paul et al. (1997) reported that one of the effects of statin drug in reducing cholesterol is stimulating the secretion of the Apo A1 by the Hep G2 cells thus enhancing the reverse transport of circulating cholesterol. Apo A-I is vital in the formation of HDL, in which due to its absence no HDL will be presented in plasma (Hiromitsu et al., 2002).

LCAT

LCAT plays a central role in HDL-mediated transport of excess cholesterol from peripheral tissues for disposal in the liver involved in RCT. During the process, cholesterol is absorbed into HDL particles and esterified with a long chain fatty acid by LCAT before being taken up to the liver and excreted in the bile (Milada and Jiri, 1999; Sander et al., 1989). Kuivenhoven et al. (1997) reported LCAT shortage will trigger to significant reduction in plasma HDL cholesterol concentration, formation of cholesterol-laden cells in various tissues, corneal opacification, premature atherosclerotic cardiovascular disease and progressive renal insufficiency. Thus, any substance that can increase LCAT is considered very beneficial in the treatment of high blood cholesterol.

The results of the LCAT are shown in Figure 3. Result revealed that supplementation of TCAE significantly increased ($p<0.05$) production of LCAT in Hep G2 cells compared to NC group. Interestingly, TCAE at 10 mg/ml exhibited significantly ($p<0.001$) higher concentration of LCAT than simvastatin drug does indicating that TCAE might have strong anti-cholesterol effect by enhancing

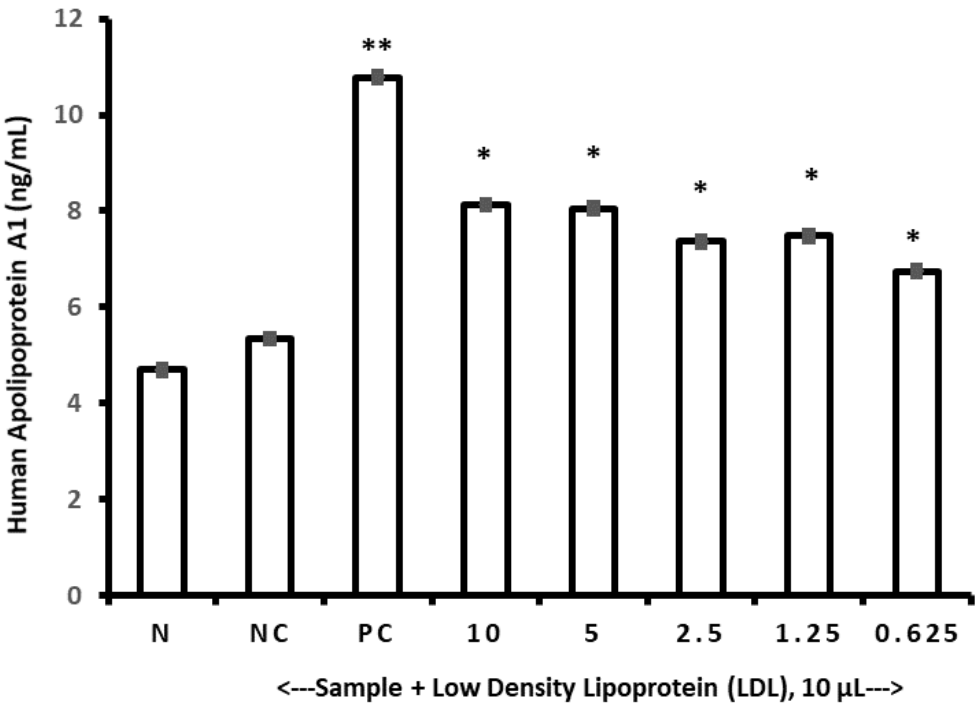

Figure 2. Graph of Human Apo A1 concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. Data expressed as mean ± SD. *Significantly different compared to NC at $p<0.05$. **Significantly different compared to NC at $p<0.01$.

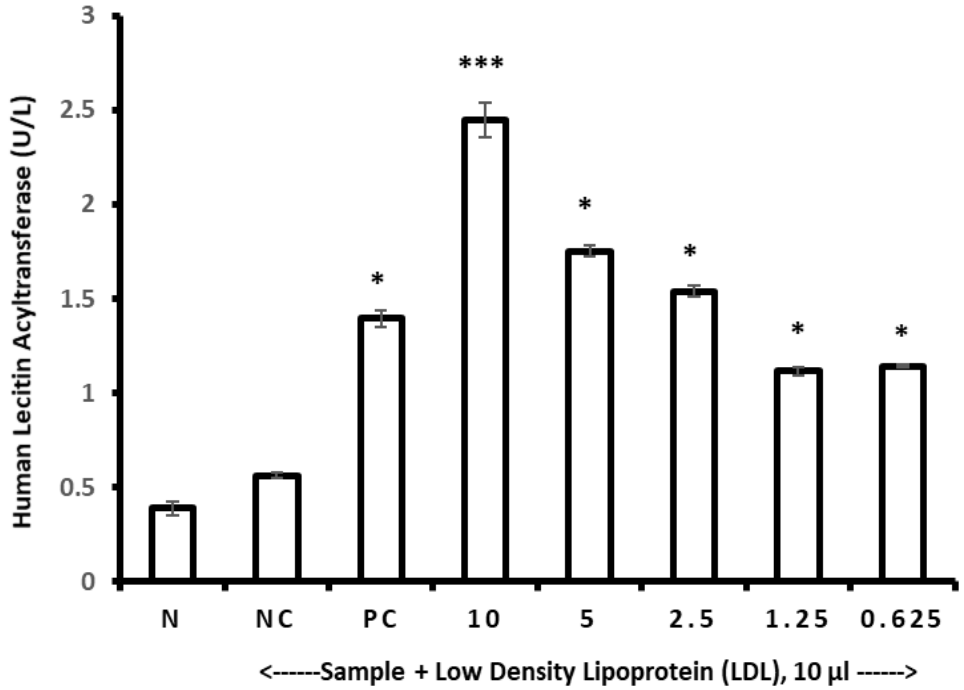

Figure 3. Graph of human LCAT concentration against treatment of samples. The samples are Simvastatin as control and different concentration of TCAE and syringin as mean ± SD. *Significantly different compared to NC at $p<0.05$. **Significantly different compared to NC at $p<0.01$. ***Significantly different compared to NC at $p<0.001$.

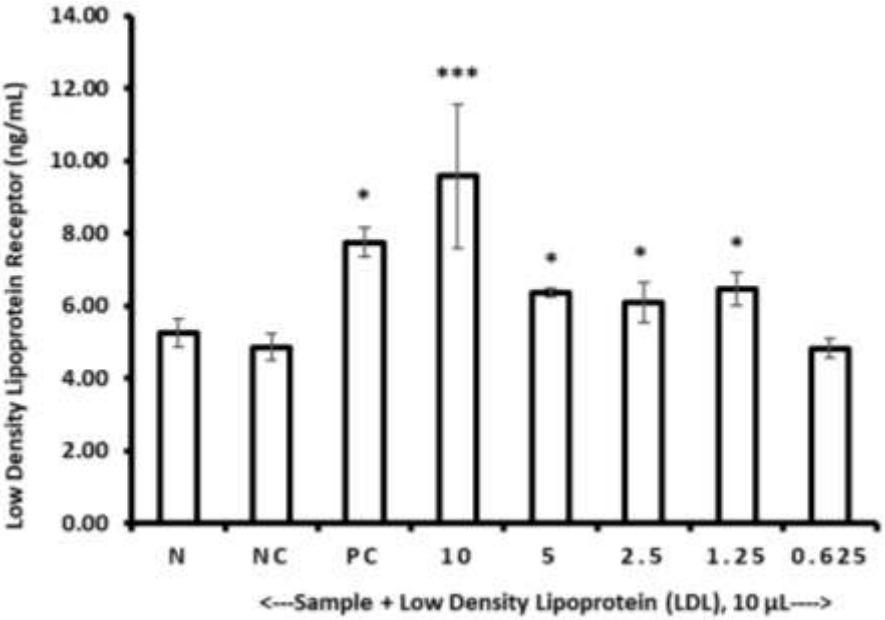

Figure 4. Graph of Human LDLR concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. *Significantly different compared to NC at $p<0.05$. *** Significantly different compared to NC at $p<0.001$.

the RCT pathway. Our finding is in line with Kumar et al. (2013), who reported that lipid metabolism disorders may increase in LDL levels of the alloxan-induced diabetic rats and consequently decrease the plasma levels of LCAT and HDL as well post-heparin lipolytic activity (PHLA). In contrast, treatment with *T. cordifolia* was able to stimulate LCAT enzyme and partially recover the level of HDL in diabetic rats.

LDLR

The results of the LDLR are shown in Figure 4. Results revealed that treatment of TCAE and simvastatin caused the increment of LDLR level in Hep G2 cell. Interestingly, TCAE at concentration of 10.0 mg/ml was able to increase the secretion of LDLR by Hep G2 cells stronger than simvastatin with the increment of 1.97 and 1.59 folds, respectively. LDL, known as bad cholesterol, is cleared from plasma by the action of the LDLR as one of the mechanism involved in RCT (Ghanya et al., 2010). Commercial available anti-cholesterol drugs such as statin and simvastatin increase the expression of LDLR and LDL uptake by liver resulting in low cholesterol level in blood plasma (Yokoyama et al., 2007; Polisecki et al., 2008).

SRB-1

SRB-1, also known as HDL receptor, functions as a key regulator of HDL metabolism (good cholesterol) and as a receiving platform of the triglyceride contents in the liver (Gillard et al., 2017). It facilitates the efflux of cholesterol from cells in peripheral tissues to HDL and mediates the selective uptake of cholesteryl esters from HDL in the liver in the RCT pathway (Van Eck et al., 2003). Therefore, any substance that can increase the concentration of SRB-1 is considered very useful in combating hypercholesterolemia. Result as presented in Figure 5 revealed that, the untreated group which was supplemented with 10 µl LDL only (NC group) exhibited low levels of SRB-1 compared to other treatment groups, indicating that high levels of LDL caused low secretion of SRB-1 in hepatocytes (Atshaves et al., 2009). SRB-1 deficiency could lead to the accumulation of HDL cholesterol within the circulation as a direct consequence of an impaired delivery to the liver, thus affect the availability of cholesterol for excretion into the bile (Van Eck et al., 2003). In contrast, administration of TCAE normalized SRB-1 concentration. Improvement of SRB-1 deficiencies through the introduction of TCAE could enhance reverse cholesterol transport in facilitating the transport of accumulated cholesterol from the plasma into the liver for excretion and thus might prove their abilities to lower the risk of cardiovascular complications.

HL

The graph of HL concentrations against treatment

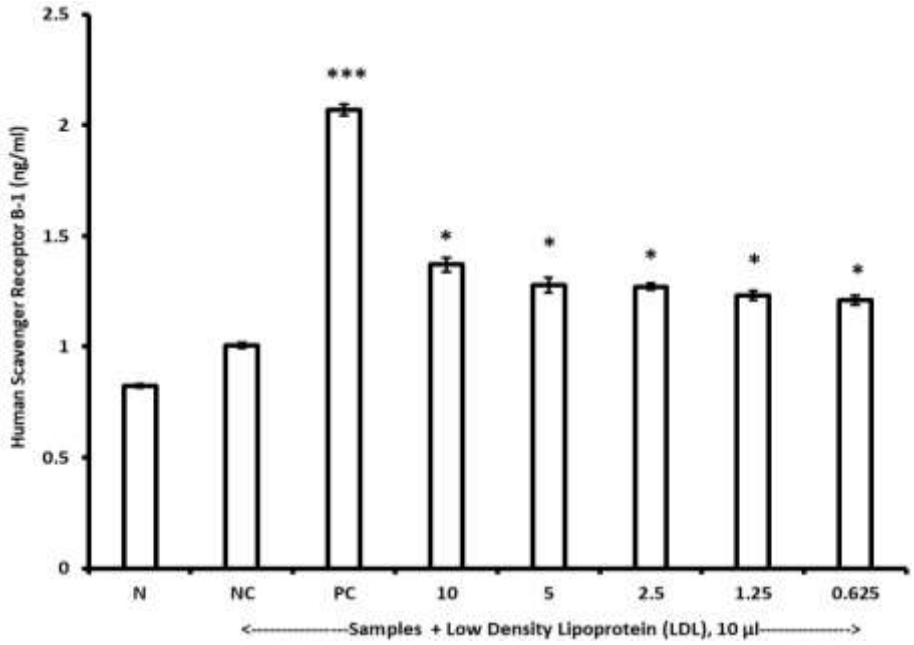

Figure 5. Graph of Human SRB-1 concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. *Significant difference compared to NC at $p<0.05$. ***Significantly different compared to NC at $p<0.0001$.

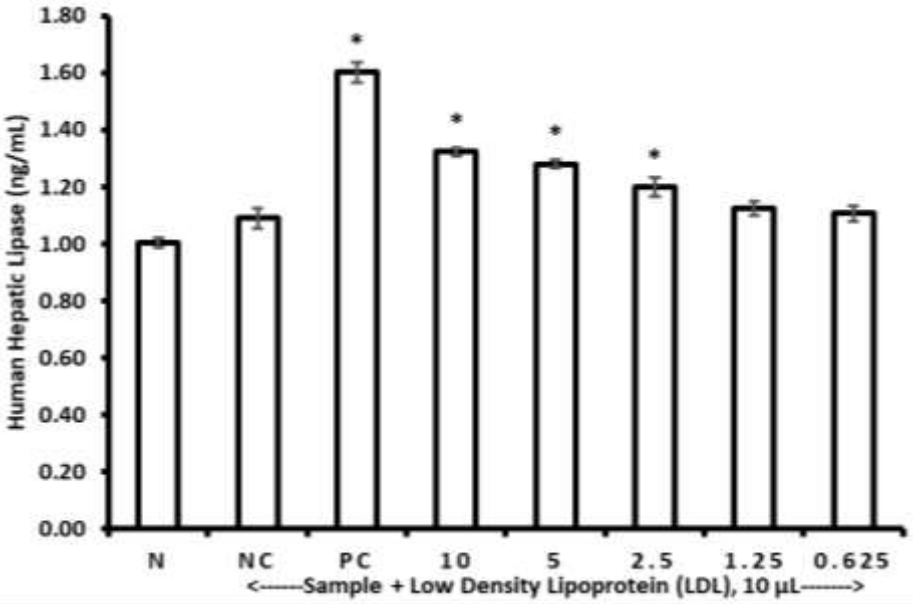

Figure 6. Graph of Human HL concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. Data expressed as mean ± SD. *Significantly different compared to NC at $p<0.05$.

samples is as shown in Figure 6. Result revealed that, supplementation of TCAE at concentration ranging from 0.625 to 10.0 mg/ml were able to promote Hep G2 cell to significantly increase ($p<0.05$) the HL production when compared with NC group. Interestingly, TCAE at 10.0, 5.0 and 2.5 mg/ml increased HL levels by which the

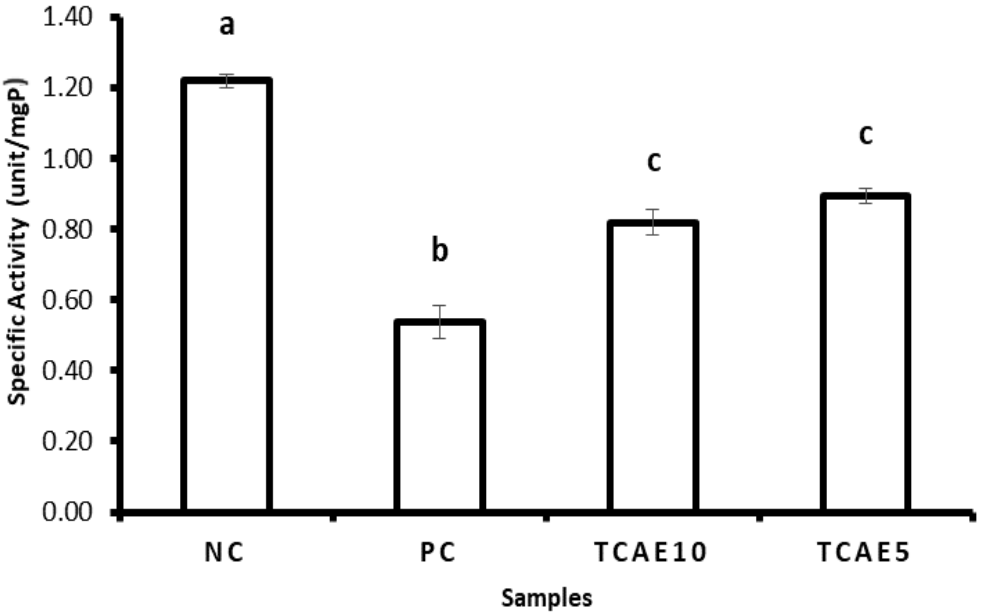

Figure 7. Graph of HMG-CoA Reductase activity against treatment of samples. The samples are Simvastatin as Positive Control (PC) and different concentration (mg/mL) of TCAE. Data expressed as mean ± SD. Data with same uppercase alphabet are not significantly different at $p<0.05$.

increment was comparable to PC group indicating a probable lipid lowering ability of TCAE in the *in vitro* system. The finding was in similar trend with *in vivo* study done by Kumar et al. (2013) who reported that the reduction of hepatic lipases in alloxan induced diabetic-dyslpidemia rats had caused hyper β-lipoproteinemia in which their reactivation by the treatment with *T. cordifolia* had played a significant role in regulation of lipoprotein metabolism back to the normal state. According to Andrés-Blasco et al. (2015), HL deficiency can trigger the increment of triglycerides and cholesterol levels in the blood resulting to the risk of developing atherosclerosis and heart disease. Therefore, high level of HL in the plasma triggered by TCAE can accelerate RCT and enhance catabolism of excess LDL from the liver.

Effects of TCAE on HMGR activity

Two-third of total cholesterol is synthesized endogenously by hepatocytes, whereas one-third of the total cholesterol is derived from diet (Kishor et al., 2007). In the liver, enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGR) plays important role in catalysing the cholesterol biosynthesis as a direct pathway in supplying cholesterol to peripheral cells (Meisel et al., 2011). Thus, it is well accepted that, one of the important approach to treat hypercholesterolemia is by inhibiting the HMGR activity as what simvastatin drug does (Farnier et al., 2012).

Result in Figure 7 showed that supplementation of TCAE at 10 and 5.0 mg/ml significantly reduced ($p<0.05$) HMGR activity in Hep G2 cells in dose dependent manner with the reduction of 32.9 and 26.7%, respectively, when compared with NC group. However, TACE ability to inhibit HMGR activity was found not comparable with that of simvastatin. This indicates that TCAE exhibited moderate inhibition of HMGR activity in Hep G2 cells which might explain the findings from the previous *in vivo* studies (Nagaraja et al., 2008; Zulkhairi et al., 2009). Hence, it is suggested that the anti-hypercholesteroleamic effects of *T. crispa* reported possibly contributed through other pathway such as RCT.

Meanwhile, the group treated with 10 µM simvastatin, showed the highest inhibition (56%) of HMGR activity when compared with NC group. This result is consistent with the study done by Bergstrom et al. (1998), who reported that atorvastatin and simvastatin caused inhibition of HMG-CoA reductase activity in Hep G2 cells. Similar findings was reported by Wilcox et al. (1999) who demonstrated that supplementation of 10 µM simvastatin decreased cellular cholesterol synthesis and CE mass in Hep G2 cell by up to 96% ($P < 0.001$) and 52% ($P < 0.001$), respectively and furthermore these inhibitors have been widely used to lower plasma cholesterol levels. Simvastatin was reported to decrease in plasma lipid levels by two different mechanisms: inhibition of HMG-CoA reductase and depression of *de novo* synthesis of PC via the cytidine diphosphate-choline pathway (Hwang et al., 2017).

Conclusions

Conclusively, the results suggest that TCAE shows strong cholesterol reducing effects demonstrated by a significant increase in molecules levels involved in reverse cholesterol transport (Apo A1, LCAT, LDL-R, SRB-1 and HL) in Hep G2 cells. The efficacy of these activities is appreciably good when compared with standard drug simvastatin. However, TCAE shows moderate effect in controlling mevalonate pathway. It could be suggested, that the pathway of TCAE action in lowering the total cholesterol possibly more on reverse cholesterol transport. However, this study was done on cell culture which has certain limitation and weakness. *In vivo* study needs to be conducted further to comprehend the anti-cholesterol effects of TCAE in the actual biological system. Besides that, it is also important to determine the chemical compounds that are involved in the up-regulation of the cholesterol metabolism.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antimicrobial evaluation of endophytic fungi extracts isolated from *Casearia sylvestris*

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Due to widespread bacterial resistance to commercial antibiotics, the search for capable substances to combating these microorganisms became a priority. In this context, the endophytic fungi gained prominence as potential producers of bioactive substances with pharmacological interest. It is considering that endophytes are still poorly studied, especially in tropical species. The antibacterial and antifungal potential of endophytic fungi associated with the medicinal plant *Casearia sylvestris* were isolated and evaluated. A total of 162 strains were obtained, among these strains, 34 were selected for antimicrobial assays, after molecular sorting with oligonucleotide (GTG)5. A total of 25 isolates showed some antifungal and / or antibacterial activity against the bacteria *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and yeasts *Candida albicans* and *Candida tropicallis*. The results show the endophytic fungi present in *C. sylvestris* have a high potential to produce bioactive compounds inhibiting pathogenic microorganisms.

Key words: Endophytic fungi, *Casearia sylvestris,* antimicrobial, secondary metabolites.

INTRODUCTION

Endophytic fungi live inside plants colonizing the internal tissues without causing immediate damage to the host. This association suggests that these microorganisms co-evolved with their hosts, presenting an intimate mutualistic relationship, where endophytes receive nutrients and protection while the plant also gains advantages in this interaction, such as greater resistance in environments of intense stress caused by biotic and abiotic factors as insects, herbivores, parasitic nematodes and phytopathogenic microorganisms, water stress and nutrient poor soil (Esposito and Azevedo., 2010; Kharwar et al., 2011; Araújo et al., 2010).

Endophytes associated with the aerial parts of plants have aroused the interest of the scientific community, especially for its potential to produce metabolites of economic interest. Endophytes inhabit a similar ecological niche occupied by phytopathogens, thus being able to control them through competition of nutrients, production of antagonistic substances, parasitizing the pathogen or even inducing the plant to develop resistance (Araújo et al., 2010). Its biotechnological use has been growing in recent decades. They are potentially useful in agriculture and industry, particularly in pharmaceuticals (Souza et al., 2004).

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Drug resistance in bacteria has become a global concern and the screening programs for new antibacterial agents are urgent and ongoing. Endophytes provide an abundant reservoir of bioactive metabolites for medicinal exploitation, and an increasing number of novel compounds are being isolated from endophytic fungi. The endophytic fungi natural products present a broad spectrum of activities such as antimicrobial (Aly et al., 2011), antioxidant (Jalgaonwala et al., 2017), immunosuppressive properties, antivirals (Strobel, et al., 2003), anticholinesterase (Aly et al., 2011), antineoplasic (Zhang et al., 2006; Shweta et al., 2010) and cytotoxic activities (Aly et al., 2011). Some studies have shown that endophytic fungi can produce many important bioactive secondary metabolites, such as Taxol (R) from the endophytic fungus *Taxomyces andreanae* and vincristine isolated from the endophytic fungus *Fusarium oxysporum*, which are important anticancer drugs.

The endophytic community may vary by host, geographic distribution, plant age, ecological and seasonal conditions, including altitude and precipitation. Usually only one or two species are dominant as endophytic in a host, while other isolates are uncommon (Arnold et al., 2003). Both the host plant and the region where the endophytes are isolated may influence the secondary metabolite production. In this context, several varieties of plants with pharmacological potentials standout such as *Casearia sylvestris* popularly known as meat leaf, guaçatonga, coffee of bush, wild coffee. It is widely distributed in Brazil and its species excel by the medicinal application. Pharmacological studies have verified in the extracts of this plant antiulcerogenic, anti-inflammatory, antiofidic and cytotoxic activities (Schoenfelder et al., 2008; Santos et al., 2013). Among microorganisms, endophytic fungi have been shown to be prosperous in the production of secondary compounds with pharmacological properties of scientific interest. However, there are still few data in literature about endophytic fungi associated with plant species. Therefore, this study aimed to isolate and evaluate the antibacterial and antifungal properties of endophytic fungi associated with *C. sylvestris* (Figure 1).

MATERIALS AND METHODS

Material collection

The material was collected in Palmas-TO, Brazil, on shores of the lake of the Lajeado hydroelectric power plant. Three leaves from each one of 20 samples of *C. sylvestris* were collected. A sample was recorded in EMBRAPA herbarium (CEN 8047).

Isolation and preservation

The collected leaves were washed previously with neutral detergent and rinsed with sterile water. They were then immersed in 70% (v/v) alcohol (1 min), 2% sodium hypochlorite (3 min) and again in sterile mQ H_2O water (2 min) for final disinfection. Subsequently, five fragments (1.0 cm in diameter) were removed from each leaf and transferred to Petri dishes containing Dextrose-Potato Agar (PDA) medium supplemented with 100 µg /mL chloramphenicol and incubated at 25°C for 60 days. After this period, the grown fungi were isolated and transferred individually to Petri dishes containing PDA and incubated at 25°C for 7 days for purification (Pereira et al., 1993). This procedure ensures the elimination of epiphytic fungi.

Grouping of endophytic fungi

DNA from all isolates was used as a template for amplification by the MP-PCR technique with microsatellite primer (GTG)5 (Lieckfeldt et al. 1993). The products of these amplifications were analyzed on 1.5% agarose gel profiles analyzed in the program PyElph 1.4 (Pavel and Vasile, 2012) with the aim of grouping more similar individuals.

Obtaining crude fungus extracts

The isolated filamentous fungi were grown in PDA at 25°C for 14 days. After this period, fungal mycelium was fragmented with the culture medium, which was subjected to extraction by maceration at room temperature using as extracting liquid a hydroethanolic solution (2:8 v/v). The macerates were filtered, and the crude extracts were evaporated at rotary evaporator to remove the solvent and then lyophilized. Extracts were diluted in 1% dimethyl sulfoxide (DMSO).

Inoculum preparation

Bacterial and fungal suspensions were standardized from a 24 h culture in Mueller Hinton Broth (MHB) for *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) bacteria. For yeasts *Candida albicans* (ATCC 10231) and *Candida tropicallis* (ATCC 13803), the Sabouraud broth was used until it reached turbidity equal to the 0.5 tube suspension of the McFarland scale (approximately 1.0×10^8 CFU / mL). The spectrophotometric reading was verified at 620 nm to confirm the concentration of microorganisms. Subsequently, a 1:10 dilution in MHB was performed, obtaining a suspension of 1.0×10^7 CFU / mL, which was used in the tests.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the microplate dilution technique (96 holes) according to the methodology described according to the M7-A6 standard of the Manual 38 Clinical and Laboratory Standards Institute (CLSI, 2008). The microplate wells were filled with 100 µL of MHB, then 100 µL of fungal extract solution was added and a serial dilution of 1000 to 7.8 µg / mL was performed. In addition, 20 µL of the microorganism suspensions were inoculated into each well of the microplates. As a positive control, chloramphenicol was used for bacterial and fluconazole tests for yeast at the same dilutions as extracts. Control of the culture medium, bacterial growth control and negative control (solvents) were also performed. The microplates were incubated in an incubator at 37°C for 24 h for bacteria and for 48 h for the yeasts; all tests were performed in triplicate.

RESULTS

Grouping of endophytic fungi

The total DNA amplification products of the 162

Figure 1. A. Example of *Casearia sylvestris*. **B.** Highlight of branches with leaves and fruits.

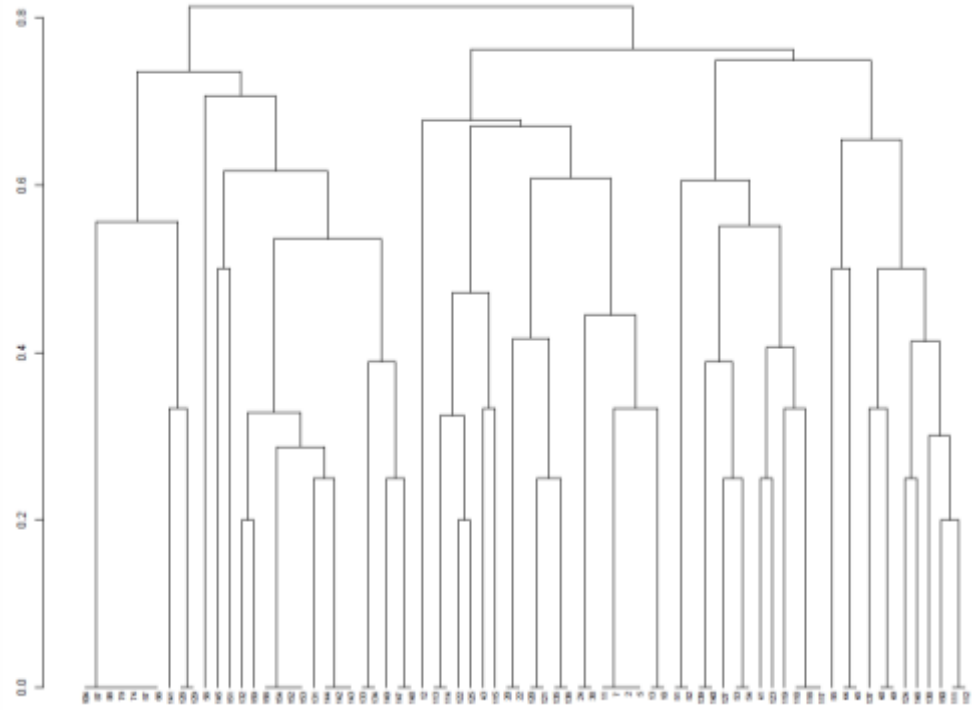

Figure 2. Phylogenetic tree generated by UPGMA method based on DNA amplification of endophytic fungi isolated from *C. sylvestris* and amplified by MSP-PCR with primer (GTG) 5.

endophytic fungus isolates obtained were analyzed and the fingerprint of each isolate was analyzed in the PyElph 1.4 program (Pavel and Vasile, 2012). A primary dendrogram was generated with all individuals. From these grouping analyzes, 34 groups were formed. A second dendrogram was generated excluding individuals with the same amplification profile, so a second dendrogram with better resolution was achieved (Figure 2). From this second grouping, 34 individuals were selected representing each of the groups formed (Table 1).

Antibacterial tests

The antimicrobial activity of ethanolic extracts from each one of 34 fungi is presented in Table 1. Among the extracts tested, 20 (58.8%) showed antibacterial activity against at least one of the four bacteria tested, 14 extracts (41.2%) did not inhibit any of the bacteria. Only one sample (P8B20) inhibited all bacteria tested. *P. aeruginosa* presented the highest resistance against the extracts tested, presenting sensitivity only for 6 (17.6%) samples (P10A2, P11B1, P11C3, P12A5, P13A1,

Table 1. Minimum inhibitory concentration (MIC) of endophytic fungi isolated from *C. sylvestris* against bacteria, *S. aureus*, *L. monocytogenes*, *E. coli*, *P. aeruginosa*.

Endophytic fungi		MIC (µg/ml)			
		Bacteria			
		S. aureus	***L. monocytogenes***	***E. coli***	***P. aeruginosa***
2	P10A2	-	-	1000	125
12	P11B1	-	-	1000	1000
13	P11C3	-	-	-	125
20	P12A4	-	-	-	-
38	P12A5	-	-	125	125
41	P13A1	-	-	125	125
43	P13A2	-	-	-	-
45	P13A4	500	500	250	-
48	P14A1	1000	1000	500	-
51	P14A5	500	1000	500	-
53	P14B1	-	-	-	-
56	P14B3	-	-	-	-
96	P14C1	500	250	125	-
104	P15A1	1000	500	500	-
112	P15A2	-	-	-	-
113	P15B2	-	-	-	-
118	P15B3	-	-	1000	-
121	P16B3	-	-	-	-
122	P16C1	-	-	-	-
124	P16C2	-	-	-	-
127	P17A1	500	250	250	-
129	P17C1	1000	-	500	-
131	P18C2	-	-	-	-
132	P20B2	-	-	-	-
133	P2B6	1000	-	-	-
136	P2C4	1000	-	1000	-
139	P3A3	-	-	-	-
143	P4C4	-	-	-	-
145	P5A3	500	-	-	-
146	P8B2	-	-	250	-
147	P8B20	250	250	125	1000
149	P8B4	-	-	1000	-
150	P8C2	-	-	1000	-
151	P8C3	-	-	-	-

* (-) There was no inhibition at the tested concentrations.

P8B20). *E. coli* presented lower resistance to the tested extracts, presenting sensitivity to 17 (50%) of the samples. The extracts (P10A2, P11B1, P12A5 and P13A1) had antibacterial action only against the Gram-negative bacteria *E. coli* and *P. aeruginosa*. The extracts (P13A4, P14A1, P14A5, P14C1, P15A1 and P17A1) inhibited Gram-positive *S. aureus* and *L. monocytogenes* and at least one Gram-negative, *E. coli*. Isolates (P15B3, P2B6, P8B4, P8C2) showed a limiting MIC in the tests (1000 µg/ml). Isolates P12A5 and P13A1 inhibited *E. coli* and *P. aeruginosa* with the lowest MIC values (125 µg/ml). All extracts tested demonstrated that antibacterial action acted with MIC between 125 and 1000 µg/ml, which demonstrates good or moderate antibacterial activity.

Antifungal tests

The antifungal activity of the ethanolic extracts from each one of 34 fungi tested was evaluated against yeasts *C.*

Table 2. Minimum inhibitory concentration (MIC) of endophytic fungi extracts isolated from *C. sylvestris* against yeasts *C. albicans* and *C. tropicallis*.

Endophytic fungi		MIC (µg/ml) Yeasts	
		C. albicans	***C. tropicallis***
1	P10A2	125	250
2	P11B1	125	1000
3	P11C3	-	-
4	P12A4	250	1000
5	P12A5	250	250
6	P13A1	-	-
7	P13A2	500	500
8	P13A4	500	125
9	P14A1	500	-
10	P14A5	500	-
11	P14B1	-	-
12	P14B3	-	1000
13	P14C1	-	-
14	P15A1	1000	-
15	P15A2	-	-
16	P15B2	-	-
17	P15B3	-	-
18	P16B3	-	-
19	P16C1	-	-
20	P16C2	-	-
21	P17A1	-	-
22	P17C1	-	-
23	P18C2	500	500
24	P20B2	250	125
25	P2B6	1000	500
26	P2C4	1000	500
27	P3A3	-	-
28	P4C4	-	-
29	P5A3	125	-
30	P8B2	500	500
31	P20B8	250	250
32	P8B4	-	-
33	P8C2	-	-
34	P8C3	-	-

* (-) There was no inhibition at the tested concentrations.

albicans and *C. tropicallis.* Of the samples tested, 12 (35.3%) presented antifungal action against the two species of yeasts, 5 extracts (14.7%) presented antifungal action against at least one of the tested yeasts and 17 extracts (50%) showed no antifungal action against yeasts tested. Both yeasts showed similar resistance to the extracts tested (Table 2). The extracts P20B2 and P20B8 inhibited the two yeasts at the lowestinhibitory concentrations tested. The sample P20B8 showed antifungal and antibacterial action for all samples tested. The extracts P12A5, P10A2 and P11B1 showed antimicrobial activity for the yeasts tested and for all Gram-negative bacteria evaluated in this work.

DISCUSSION

The antimicrobial potential of endophytic fungi is associated with their metabolic potential and their ability to produce a great diversity of bioactive molecules, whose main function is to protect the plant from pathogens (Tan and Zou, 2001; Strobel et al., 2003). Many of these bioactive molecules isolated from endophytic belong to various structural classes such as

alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids (Yu et al., 2010).

Li et al. (2012) reported the presence of acidic, antifungal agent isolated from the endophytic fungi *Pestalotiopsis microspora* and *Monochaetia* sp. present in plant *Torreya taxifolia.* Jadulco et al. (2002) demonstrate the presence of cytosquirins antifungal agent isolated *Curvularia lunata*, present in the plant *Niphates olemda.* Weber et al. (2004) reported the presence of Phomol, which has cytotoxic, antifungal, antibacterial and anti-inflammatory activity and isolated from the endophytic fungi *Phomopsis* spp.

In the present study, 32.3, 50 and 47.1% of the extracts showed antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*, respectively. These data contrast those of Guimaraes et al. (2008) which examined 39 endophytic fungi extracts and found 5.1, 25.6 and 64% of the extracts inhibited *S. aureus*, *E. coli* and *C. albicans*, respectively, indicating that endophytes of *C. sylvestris* have a similar antibacterial potential as endophytic isolates of *Viguiera arenaria* and *Tithonia*, although it has a lower antifungal action. 11.8% of the tested extracts inhibited only Gram-positive bacteria, a data that corroborate with studies conducted by Ratnaweera et al. (2014) and Philips et al. (1989) which identified an active compound corresponds to the tetramic acid derivative known as equisetin isolated from various species of *Fusarium* spp.; they also demonstrated antimicrobial activity against Gram-positive bacteria such as *B. subtilis*, *S. aureus* and MRSA, but showed no activity against Gram-negative bacteria such as *E. coli*, *P. aeruginosa* and *C. albicans* pathogenic fungi.

The antifungal tests demonstrated that 35.3% of the extracts inhibited *C. albicans* and *C. Tropicallis*. Similarly, Strobel et al. (1999), in studies carried out with extract of fungus *Cryptosporiopsis quercina* isolated from *Tripterigeum wilfordii,* showed antifungal activity against *C. albicans*, which reveals the potential of endophytic fungi to combat this yeast.

In this work, the extracts of 34 endophytic fungi obtained from *C. sylvestris* were tested; 25 of them demonstrated some antibacterial and / or antifungal activity against different Gram positive, Gram negative and yeast microorganisms, by the microdilution technique in plaques; the most recommended method for this determination. The results demonstrate the antibacterial and antifungal properties of these endophytes, revealing the potential of their ethanolic extracts and its application in production of bioactive antimicrobial compounds.

Conclusion

This study reveals that *C. sylvestris* hosts a rich community of endophytic fungi with antimicrobial potential. However, complementary studies are being carried out to identify which secondary components are present in each of the extracts tested.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of *Ephedra foeminea* active compounds on cell viability and actin structures in cancer cell lines

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***Ephedra* is likely one of the oldest medicinal plants still currently in use. In folk medicine, extracts of *Ephedra foeminea* are commonly used to treat cancer patients. In relation to its traditional use, the aim of the present study was to determine the cytotoxic activity *in vitro* of *E. foeminea* extracts on cancer and non-cancer cells. Cell viability was determined using XTT assay, induction of apoptosis by cell sorting and caspase-3 inhibition, and the effects on cell cytoskeleton structure were detected using cell transfection utilizing different cytoskeleton markers. Chemical profiling, analysis of active extracts and identification of compounds was done using high pressure liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GCMS). *E. foeminea* leaf ethanol extract and *E. foeminea* fruit juice reduced cancer cell viability *in vitro*, whereas the water extract reduced cytotoxic activity in all cell lines. The extract's cytotoxic activity was conveyed at least partially via the induction of caspase 3-dependent cell apoptosis, and enhanced by the addition of Taxol. Both *E. foeminea* ethanol leaf extract and fruit juice affected actin-stained but not tubulin-stained filaments. Ethanol extract promoted the formation of invadopodia-like structures and fruit juice promoted the formation of large focal adhesion points in the treated cells. Active sub-fractions of *E. foeminea* extracts were found to contain several compounds including trans-sinapyl alcohol and trans-sinapaldehyde derivative.**

Key words: *Ephedra foeminea*, plant extract, cancer cells, actin, invadopodia, apoptosis.

INTRODUCTION

Ephedra is a ubiquitous genus of gymnosperm shrubs that grow in temperate and subtropical regions usually on shores or in sandy soils under direct sunlight throughout North and Central America, Europe, Africa and Asia

(Ickert-Bondmet et al., 2009). It is probably one of the oldest medicinal plants still currently in use, featured prominently in traditional Chinese medicine for over 2000 years (Abourashed et al., 2003). The main species used in Chinese medicine is *Ephedra sinica* Stapf, traditionally known in Chinese as 'ma huang' (Gurley et al., 1998). Premodern Chinese, Native Americans and Mormons boil the green leaves in water and serve this extract as tea to treat respiratory congestion and asthma (Normile, 2003).

The clinical interest in the folk use of *Ephedra* increased during the 20th century especially for the treatment of cancer. Importantly, an *Ephedra foeminea* decoction (that is, water extract) has been used widely by cancer patients to treat their ailments (Ben-Arye et al., 2016). Recently, in a research on the use of complementary and alternative medicines in Palestinian populations, it was reported that 68% of breast cancer patients were supplementing treatment with herbal remedies, with *E. foeminea* as the primary ingredient (45.7%) (Ali-Shtayeh et al., 2016). Moreover, *E. foeminea* only recently became one of the most commonly utilized plants by the Palestinian population- from 0.0% in 2011 to 55.2% in 2014, primarily due to local media reporting of *E. foeminea* decoctions as an effective herbal remedy for cancer patients (Ali-Shtayeh et al., 2016). These water extracts are generally prepared by boiling the leaves for an hour (Ben-Arye et al., 2016), although in some cases, the fruits are consumed as well. Reports show that some breast cancer patients even completely replace modern chemotherapy treatment with *E. foeminea* herbal extracts (Ben-Arye et al., 2016). Hence, it is extremely important to examine the effectiveness of *E. foeminea* against cancer in general, and breast cancer in particular.

In the last 15 to 20 years, the use of *Ephedra* has been restricted due to potentially hazardous effects (Abourashed et al., 2003). Indeed, the aerial parts of different *Ephedra* species contain active alkaloids (Yeung, 1980), including the phenylpropylamino alkaloids ephedrine and pseudoephedrine. Ephedrine is a sympathomimetic agent (Astrup et al., 1995; Kobayashi et al., 2003) and its consumption may lead to increased cardiac rate and contractility as well as overstimulation of the central nervous system (Abourashed et al., 2003; Krizevski et al., 2012). However, *E. foeminea* species specifically lacks both ephedrine and pseudoephedrine (Abourashed et al., 2003; Krizevski et al., 2012).

In this paper, the efficacy of *E. foeminea* extracts against cancer cells *in vitro* in relation to its folk-use was examined. The effect of different extracts of *E. foeminea* (leaf ethanol extract vs. water extract) and fruit juice on viability of cancer and non-cancer cells was evaluated and reported.

MATERIALS AND METHODS

Water extraction (decoction)

Leaves from male and female plants of *Ephedra foeminea* were collected in the Samaria Mountains (coordinates 32.087661° N, 35.274732° E WGS84) in Central Israel. The soils in the region are *terra rossa*; plants were collected during August 2016 (temperatures ranged from 22to 24°C) when plants were flowering. Botanical samples were verified by Dr. Jotham Ziffer-Berger, Director of the Herbarium of the National Natural History Collections, The Hebrew University of Jerusalem, Israel. *Ephedra* extracts were prepared according to instructions received from Y. Sharvit, a local herbalist. Leaves were weighed in a ratio of 43:7.8 g of male to female plants. To the leaves, 1 L of double distilled water (DDW) was added and the mixture was placed in a closed cooking pot. The mixture was brought to boil on a heating plate at medium heat; then reduced and left to simmer at a lower temperature for 2 h. After simmering, the pot was removed from the heating plate and left to cool at room temperature. The extract was then filtered using a 2.5 ml syringe with a 0.22 µm teflon filter and stored at -20°C. Diluted extract for cell treatment contained 30% of the extract and 70% of Dulbecco's Modified Eagle Medium (DMEM, Biological Industries [BI] Ltd., Cat # 01-055-1A, Kibbutz Beit-Haemek, Israel).

Fruit juice preparation

Ephedra fruits were prepared according to the instructions received again from Y. Sharvit. Thirty grams of fruit were crushed using a mortar and pestle, producing 6 ml of *Ephedra* fruit juice. The juice and crushed material was transferred to 50 ml falcon tubes and placed in a centrifuge at 6500 rpm for 5 min. The supernatant was then stored at -20°C. The homogenous solution was filtered using a 2.5 ml syringe with a 0.45 µm teflon filter and diluted with 1 L of DDW (0.6% v/v). For higher concentrations, the volume of DDW was adjusted accordingly.

Ethanol extraction

Ethanol extraction was developed in the laboratory based on the protocol of Nagappan (2012). Two grams of *E. foeminea* leaves (from male and female plants at the same ratios as mentioned earlier) were crushed using a mechanical grinder and then transferred to a 50 ml falcon tube. 20 ml of 70% ethanol (EtOH) was added and the mixture was incubated overnight at 28°C with shaking at 180 rpm. The following day, the tube was centrifuged for 5 min at 2500 rpm and the supernatant was removed under vacuum in a rotary evaporator and stored at -20°C. The extract was reconstituted by adding 160 µl of 70% EtOH to the dried sample and incubated at room temperature for 10 to 15 min. One milliliter of DDW was added to the tube. Following vortex, the homogenized solution was filtered using a 2.5 ml syringe with a 0.45 µm teflon filter. The resulting filtrate was used for the experiments described below.

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Heat inactivation of *E. foeminea* ethanol extract and fruit juice

Extracts (ethanol and fruit juice) were heated in order to examine the possible presence of heat-labile components. Heat treatment of the extracts was carried out by heating the filtered extract of each sample for 1 h at 90°C in a closed tube and left to cool for 2 h.

Cell culture

Human (*Homo sapiens*) cell lines used in this study included: MDA-MB-231-mammary gland/breast cells derived from metastatic site (ATCC® HTB-26™); A549 lung carcinomatous cells (ATCC® CRM-CCL-185™); HaCaT, keratinocytes from histologically normal skin (Creative Bioarray CSC-C8977H); HCT116, epithelial colorectal carcinoma cells (ATCC® CCL-247™). Cells were grown at 37°C in a humidified 5% CO_2- 95% air atmosphere. All tissue culture media and serum were purchased from Biological Industries (BI) Ltd., Israel. MDA-MB-231, A549 and HaCaT were maintained in DMEM, and HCT116 were maintained in McCoy's 5A medium. Both media were supplemented with 10% FBS, 1% Penicillin-Streptomycin Solution, 1% L-glutamine and 0.002% plasmocin.

XTT indirect viability and caspase-3 dependent assays

Cells were seeded into 96-well plates at a concentration of 10,000 cells per well in triplicate in DMEM. The following day, DMEM was replaced with fresh medium containing either plant extracts, solvents used for extraction, or only fresh medium for control. Cells were incubated with the different treatments for 2 days and then 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT) reduction was used to quantify viability according to the manufacturer's instruction (BI, Kibbutz Beit-Haemek, Israel). Cells were incubated with XTT reagent for 2 h at 37°C in a humidified 5% CO_2 – 95% air atmosphere. Absorbance was recorded by a photometer SPEKTRAFluor Plus (Tecan, Salzburg, Austria) at 490 nm with 650 nm reference wavelength. Cell survival was estimated from the equation: % cell survival = 100 × (At-Ac) (treatment) / (At-Ac) (control), where At and Ac are the absorbencies (490 nm) of the XTT colorimetric reaction in treated and control cultures, respectively, minus non-specific absorption measured at 650 nm. Absorbance of medium alone was also deducted from specific readings. Dose-effect curves were determined for the extracts. For dose response assays, data points were connected by non-linear regression lines of the sigmoidal dose-response relation. GraphPad Prism (Version 6 for Windows, GraphPad Software Inc., San Diego, USA) was employed to produce dose-response curves and IC50 doses for ethanol extract and fruit juice on MDA-MB-231 and HaCaT cells by performing nonlinear regression analysis.

In order to test whether the cell death events observed are mediated by caspase-3 activity Z-VAD-FMK (N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone. Abcam, Cat # ab120382, Cambridge, United Kingdom), a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases was used. A final concentration of 100 µM of the caspase inhibitor was added to treated wells to examine the differences in cell proliferation and viability in the presence of Z-VAD-FMK. Cell proliferation and viability was determined by comparing those treated with *E. foeminea* extracts with or without Z-VAD-FMK.

For all experiments, a DDW control (30% v/v water in XTT reaction mix) and ethanol control (0.17% v/v ethanol in XTT reaction mix) were tested and showed no effect (data not shown).

Actin and tubulin staining

For actin and tubulin staining, 10,000 cells in a total volume of 500 µl were seeded on confocal plates. Plates were incubated at 37°C in a humidified 5% CO_2-95%. After 24 h, in order to transfect the cells, the medium in each plate was replaced with a fresh medium (500 µl) containing 5 µl of CellLight Actin-RFP (Life Technologies, Cat # C10502, Eugene, Oregon, USA) or Tubulin-GFP (Life Technologies, Cat # C10509, Eugene, Oregon, USA) and plates were incubated under the same conditions for 16-24 h. Successful transfection was determined by confirming fluorescence in 1-2 plates, then cells were washed 3 times with 1.5 ml of DMEM and incubated under the same conditions. After 2 h, the medium was replaced with 700 µl of fresh medium containing the appropriate treatment and plates were incubated for 90 min under the same conditions. Fluorescence intensity of red/green fluorescent proteins (RFP/GFP) were examined using live-cell imaging and photographed with a Leica TCS SP8 laser scanning confocal microscope (Germany) with LAS_X software, equipped with an OPSL 488 nm laser for GFP excitation and OPSL 552 nm for RFP excitation and HC PLAPO 63X 1.2 N.A. objective.

Annexin V/PI staining

Apoptosis was assessed using Alexa Fluor® Dead Cell Apoptosis Kit with Annexin V and PI (Life Technologies, Cat # V13241, Eugene, Oregon, USA). Staining was done according to manufacturer instructions. In brief, cells were seeded in 6-well plate culture dishes at density of 5 x 10^5 cells per well in DMEM. The following day, the medium was replaced with medium containing *E. foeminea* extracts at the desired concentrations (as indicated below). Treated samples were incubated for 90 min at 37°C in humidified 5% CO_2–95% air atmosphere. Cells in each well were collected separately using trypsin. Then tubes were centrifuged for 8 min in 1400 rpm and cells were resuspended and washed twice with 1 ml of PBS. The cells in each sample were counted. If necessary, the number of cells was adjusted to a concentration of 2 x 10^6 cells/ml in 1X Annexin binding buffer. Otherwise, cells were resuspended in 200 µl of 1X Annexin binding buffer and transferred to FACS tubes. Cells were stained using 5 µl of Alexa Fluor® 488 or FITC solution and 1 µl of propidium iodide (PI) working solution followed by incubation at room temperature for 15 min in the dark. Then 400 µl of Annexin V binding buffer were added to each tube and flow cytometry was performed using a GALLIOS flow cytometer. Cells were considered to be apoptotic if they were Annexin V+/PI- (early apoptotic) and Annexin V+/ PI+ (late apoptotic). Live cells were Annexin V-/PI-.

RNA extraction, cDNA synthesis and quantitative PCR (qPCR)

Cells were seeded into a 6-well plate at a concentration of 2,000,000 cells/ml per well. After 24 h incubation at 37°C in a humidified 5% CO_2–95% air atmosphere, cells were treated with ethanol extract at a dilution of x5 for 1.5 and 3 h. Non-treated cells or cells treated only with ethanol served as controls. Cells were harvested and total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. The RNA pellet was dissolved in nuclease-free sterile water and RNA was purified and concentrated using an RNeasy MiniElute Cleanup Kit (Qiagen, USA) as per the manufacturer's instructions. For cDNA synthesis, 2.5 µg of total RNA and 0.1 µM of random hexamer primers (Promega, USA) were heated for 5 min at 65°C and snap-chilled on ice. The following components were added to the reaction mixture: 0.2 mM dNTP mixture (Invitrogen, USA), 200 U of superscript II-reverse transcriptase (RT) enzyme (Invitrogen, USA), 40 U of Ribolock RNAse inhibitor (Thermo Fisher-Scientific), reverse transcriptase (RT) buffer (1x final concentration), and nuclease-free sterile water (VWR, Amersco Life Sciences, OH, USA) to a reaction volume of 21 µl. The reaction was incubated at 42°C for 60 min

followed by incubation at 70°C for 10 min. qPCR was used to determine the gene transcription of matrix metallopeptidase 9 (*MMP9*) in treated and non-treated MDA-MB-231 cells as described above. Expression of MMPs characterizes cells undergoing induction of invadopodia, whereas MMP9 is produced mainly by the MDA-MB-231 cells and contributes to metastatic progression (Mehner et al., 2014). The qPCR was performed using components supplied in the KAPA SYBR FAST qPCR kits (Kapa Biosystems, USA) and genes specific primers. For *MMP9* (GenBank accession no. NM_004994.2), the following primers were used: (forward) 5'-TTGACAGCGACAAGAAGTGG-3' and (reverse) 5'-TCACGTCGTCCTTATGCAAG -3'. *GAPDH* served as the reference gene with forward (5'- CAGCCTCAAGATCATCAGCA-3') and reverse (5'- TGTGGTCATGAGTCCTTCCA-3') primers. The reaction mixture consisted of the following components: 2x Master Mix with integrated antibody-mediated hot start, SYBR-Green I fluorescent dye, $MgCl_2$, dNTPs, stabilizers, 2 µl of the template, and PCR-grade water to a final volume of 10 µl. The qPCR analysis was carried out on a Rotor-Gene 6000 instrument (Corbett-Qiagen, Valencia, CA, USA) according to the following program: 3 min at 95°C, followed by 49 cycles of 95°C for 3 s, 60°C for 20 s and 72°C for 1 s. The threshold cycle (Ct) was calculated by the Rotor-Gene 6000 instrument software. The values of the steady-state level of gene transcripts were determined by the $2^{-\Delta\Delta CT}$ method (Arocho et al., 2006), as a ratio between target gene (*MMP9*) versus the reference gene (*GAPDH*) and/or treated cell samples versus non-treated cells. A value above or below 1 represents an increase or decrease respectively in the steady-state level of gene transcripts for the examined conditions. Means ± SE (n = 3) were calculated for three biological replicates for each examined treatment.

High pressure liquid chromatography (HPLC) and fractionation

The concentrated *Ephedra* fruit juice, ethanol extract and water extract were filtered through 0.45 µm syringe filters. 350 µl of sample was loaded in HPLC for profiling and separation (Snyder et al., 2012). The separation of the sample was carried out with Varian Prostar HPLC system coupled with Varian 410 Autosampler, 210 pump, 320 UV/Vis detector. The separation was performed on a Purospher RP-18 endcapped column (250 mm × 4.6 mm I.D.; Merck KGaA, Darmstadt, Germany) with a guard column (4 mm × 4 mm I.D.). Solvent gradients were formed by varying the proportion of solvent A (0.1% acetic acid in DDW) to solvent B (methanol) with the flow rate of 1.0 ml min^{-1}. Initially, solvent B was maintained at 10% for 10 min and then increased to 55% in 5 min. From 55%, solvent B increased to 65% in 25 min and then maintained at 65% for 5 min and then reduced to 10% in 5 min and equilibrated for 5 min. Compound peaks were detected with two different wavelengths of 280 and 320 nm. Fractions were collected every 3 min from a total run of 30 min. Fractions were then dried using vacuum drying and lyophilization. After drying, fractions were taken for XTT assays as described above.

Sub-fractions from Fraction 7 of the ethanol extract were obtained using solvent gradients formed by isocratic proportion of 60% from solvent A and 40% of solvent B with a flow rate of 0.5 ml min^{-1} for 25 min, and were collected based on peaks. Sub-fractions were then dried using vacuum drying and lyophilization. After drying, sub-fractions were taken for XTT assays as described above.

Gas chromatography-mass spectroscopy (GCMS)

Sub-fractions 7.1 to 7.9 of fraction 7 were dried using vacuum drying and lyophilization. After drying, samples were reconstituted in 100 µl of 100% EtOH and then loaded into the GCMS for analyses (Halket et al., 2004). The analysis was carried out using an Agilent 7890B gas chromatograph coupled to a 5977A mass spectrometer (electron multiplier potential 2 KV, filament current 0.35 mA, electron energy 70 eV, and the spectra were recorded over the range m/z 40 to 500). An Agilent 7683 autosampler was used for sample introduction. 1 µl of each sample was injected to the GCMS using a 1:10 split ratio injection mode. Helium was used as a carrier gas at a constant flow of 1.1 ml s^{-1}. An isothermal hold at 50°C was kept for 2 min, followed by a heating gradient of 6°C min^{-1} to 300°C, with the final temperature held for 4 min. Solvent delay of 3 min was applied. A 30 m, 0.25 mm ID 5% cross-linked phenylmethyl siloxane capillary column (HP-5MS) with a 0.25 µm film thickness was used for separation and the injection port temperature was 200°C. The MS interface temperature was 280°C. Peak assignments were carried out with the aid of library spectra (NIST 14.0) and compared with published data and MS data obtained from the injection of standards purchased from Sigma-Aldrich.

Statistical analysis

Results are presented as mean + SE of replicate analyses and are either representative of or include at least two independent experiments. Means of replicates were subjected to statistical analysis by Tukey-Kramer test ($P \leq 0.05$) using the JMP statistical package and considered significant when $P \leq 0.05$.

RESULTS

Reduction of cell viability of cancer cells by *E. foeminea* extracts

The *E. foeminea* leaf ethanol extract and fruit juice significantly reduced MDA-MB-231, HCT116 and HaCaT cell viability (Figure 1A, B and D, respectively). However, at these concentrations, they were not effective on A549 cell viability (Figure 1C). Water extract was the least active of the extracts on the different cell lines, and even at a relatively high concentration (30% v/v extract in XTT reaction mix) lacked significant activity on A549 and HaCaT cell viability (Figure 1C and D, respectively).

Fruit juice was more effective on MDA-MB-231 than HaCaT cell lines with IC50 0.54 ± 0.07 and 0.69 ± 0.05%, respectively (Figure 2A). Ethanol extract was also more effective on MDA-MB-231 than HaCaT cell lines with IC50 0.57 ± 0.02 and 0.75 ± 0.01 mg/ml, respectively (Figure 2B).

The activity of *E. foeminea* fruit juice and ethanol extract on cancer cell viability is heat sensitive

Heat inactivation (90°C for 1 h) of *E. foeminea* fruit juice led to a significant reduction in activity in 1.5, 0.6 and 0.4% concentrations (v/v) (Figure 3A). Heat treatment of the ethanol extract affected activity to a much lesser extent; a significant reduction in activity was recorded in the 2.0 mg/ml concentration and to some extent, in the 0.8 mg/ml concentration (Figure 3B).

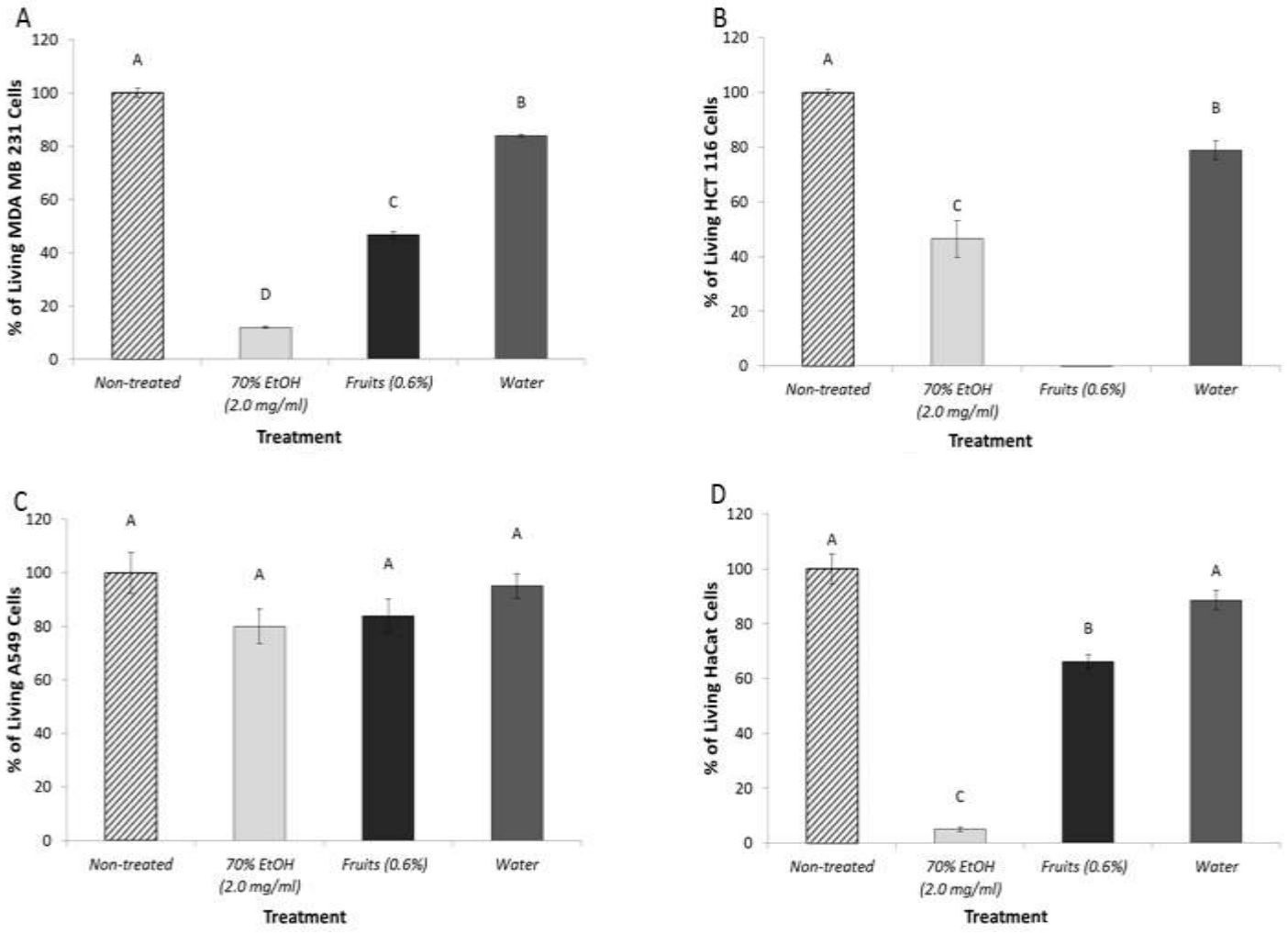

Figure 1. Effects of *E. foeminea* water and ethanol extracts and fruit juice on cell viability of (A) MDA-MB-231 breast cancer, (B) HCT 116 colon cancer, (C) A549 lung cancer and (D) HaCaT non-cancerous skin cells, determined by XTT viability assay. For each assay 10,000 cells per well were seeded in triplicate in normal growing media. Treatments were applied after 24 h and the cells were then incubated for 48 h. XTT reduction was used to quantify viability; absorbance was recorded by a photometer at 490 nm with 650 nm of reference wavelength. Cell survival was estimated from the equation: % cell survival = 100 × (At − Ac), where At and Ac are the absorbencies (490 nm) of the XTT colorimetric reaction in treated and control cultures, respectively, minus non-specific absorption measured at 650 nm. Ethanol extract (2 mg/ml extract in XTT reaction mix), fruit juice (0.6% v/v extract in XTT reaction mix) and water extract (30% v/v extract in XTT reaction mix). Levels not connected by same letter are significantly different. 70% EtOH, 70% ethanol extract of leaves; Fruit, fruit juice; Water, water extract of leaves.

The activity of *E. foeminea* ethanol extract and fruit juice on cancer cell viability as enhanced by Taxol

The addition of Taxol at a concentration of 10 nM (Mayzlish-Gati et al., 2015) to fruit juice at 0.6 and 0.4% concentration (v/v) showed reduced cell viability in comparison with activity of Taxol only or fruit juice only (in the corresponding concentrations) on cell viability (Figure 3A). Addition of Taxol at a concentration of 10 nM to the ethanol extract at concentration of 0.8 mg/ml showed reduced cell viability in comparison with the activity of Taxol only or ethanol extract only (in the corresponding concentration) on cell viability (Figure 3B).

Induction of cell death via apoptosis by *E. foeminea* extracts

Cell sorting by FACS based on Alexa Fluor® 488/Annexin V staining showed that treatment with high concentrations of the two different extracts, *E. foeminea* ethanol extract and fruit juice, lead respectively to a large proportion of cells that are in early (4.9 ± 0.7 and 43.3 ± 2.1) or late (68.2 ± 4.4 and 46.6 ± 3.8) apoptosis in comparison with the non-treated cells (FACS sorted at Q4 or Q2, respectively) (Figure 4A). The addition of Z-VAD-FMK caspase inhibitor I to *E. foeminea* ethanol extract and fruit juice led to a significant decrease in

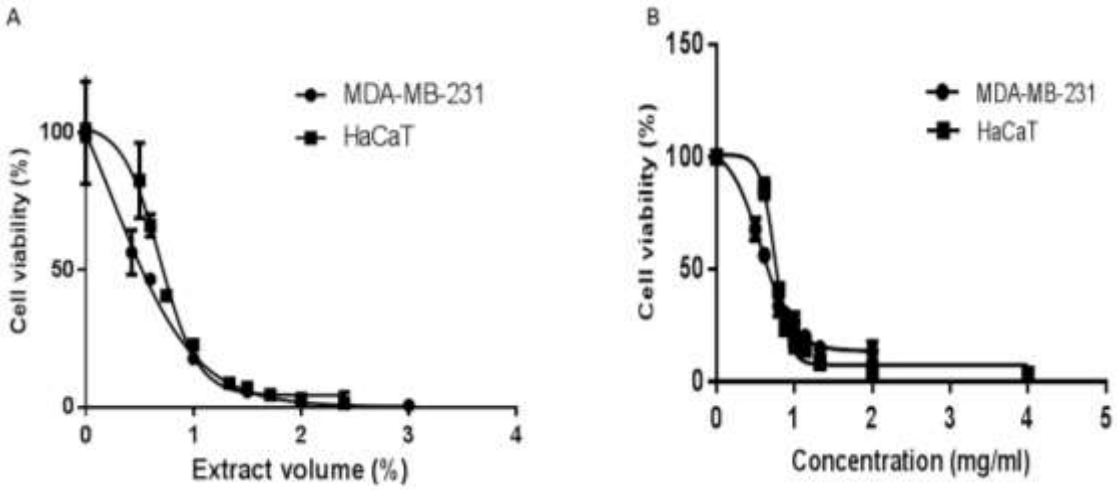

Figure 2. Dose-effect curves of (A) *E. foeminea* fruit juice and (B) *E. foeminea* ethanol extract on viability of MDA-MB-231 breast cancer and HaCaT non-cancerous skin cells, determined by XTT. For dose response assays, data points were connected by non-linear regression lines of the sigmoidal dose-response relation. GraphPad Prism was employed to produce dose-response curve and IC50 doses for ethanol extract and fruit juice were determined by nonlinear regression analysis.

activity on cancer cell viability (Figure 4B).

Effect of *E. foeminea* extracts on actin and tubulin filaments

Staining for actin and tubulin was carried out to determine the effect of *E. foeminea* ethanol extract, water extract and fruit juice on the organization of cytoskeleton filaments. Treatment of MDA-MB-231 cells with *E. foeminea* ethanol extracts (8.0 mg/ml for 90 min) caused reduction in focal adhesion points and induced formation of actin structure similar to invadopodia, characterized by bright points of actin staining distributed in MDA-MB-231 cells (Figure 5). Exposure of MDA-MB-231 cells to *E. foeminea* fruit juice (6% v/v, for 90 min) led to reduction in actin filaments and formation of what appears to be large focal adhesion points, whereas water extract did not show any effect on the actin filaments (Figure 5). No change in tubulin filaments were apparent following treatment with the *E. foeminea* extracts or fruit juice (Figure 5). Together, these results suggest that *E. foeminea* fruit juice and ethanol extract affect the organization of actin-stained but not tubulin-stained filaments. Despite their effect on actin, in a 2-D migration assay, *E. foeminea* extracts did not show significant effect on the cell migration of MDA-MB-231 breast cancer cells as compared to the control (Supplementary Figure 1).

Induction of matrix metallopeptidase 9 (*MMP9*) expression by *E. foeminea* ethanol extract

The steady state level of matrix metalloproteinases 9 (*MMP9*) mRNA in MDA-MB-231 cells was significantly increased by 6.96 ± 1.00 fold following treatment with *E. foeminea* ethanol extract.

Chemical profiling and analysis of *E. foeminea* extracts

Chemical profiling of the active *E. foeminea* ethanol extract and fruit juice was carried out by separating the chemical constituents of ethanol extracts and fruit juice in HPLC based on the multistep gradient program described in the materials and methods section. Subsequently, activity of the fractions on cell viability was tested using the XTT assay. The results demonstrated that Fraction 7 in both ethanol extract and fruit juice have high activity and significantly reduced MDA-MB-231 cell viability (Figure 6). However, the activity of other fractions was different between the two extracts. Fractions 6 and 8 had high activity in the ethanol extract, but only medium to low activity in the fruit juice. Fraction 2 also had high activity in the fruit juice, and medium activity in the ethanol extract (Figure 6).

Sub-fractionation of the active ethanol Fraction 7 in both *E. foeminea* ethanol extract and fruit juice was carried using the isocratic program described in the materials and methods section. Subsequently, activity of the sub-fractions on cell viability was tested using XTT assay. The results demonstrate that several sub-fractions, especially 7.2, 7.3 and 7.5, had high activity and significantly reduced MDA-MB-231 cell viability (Figure 7).

The active sub-fractions were analyzed for identification of compounds using GCMS. Results are presented in

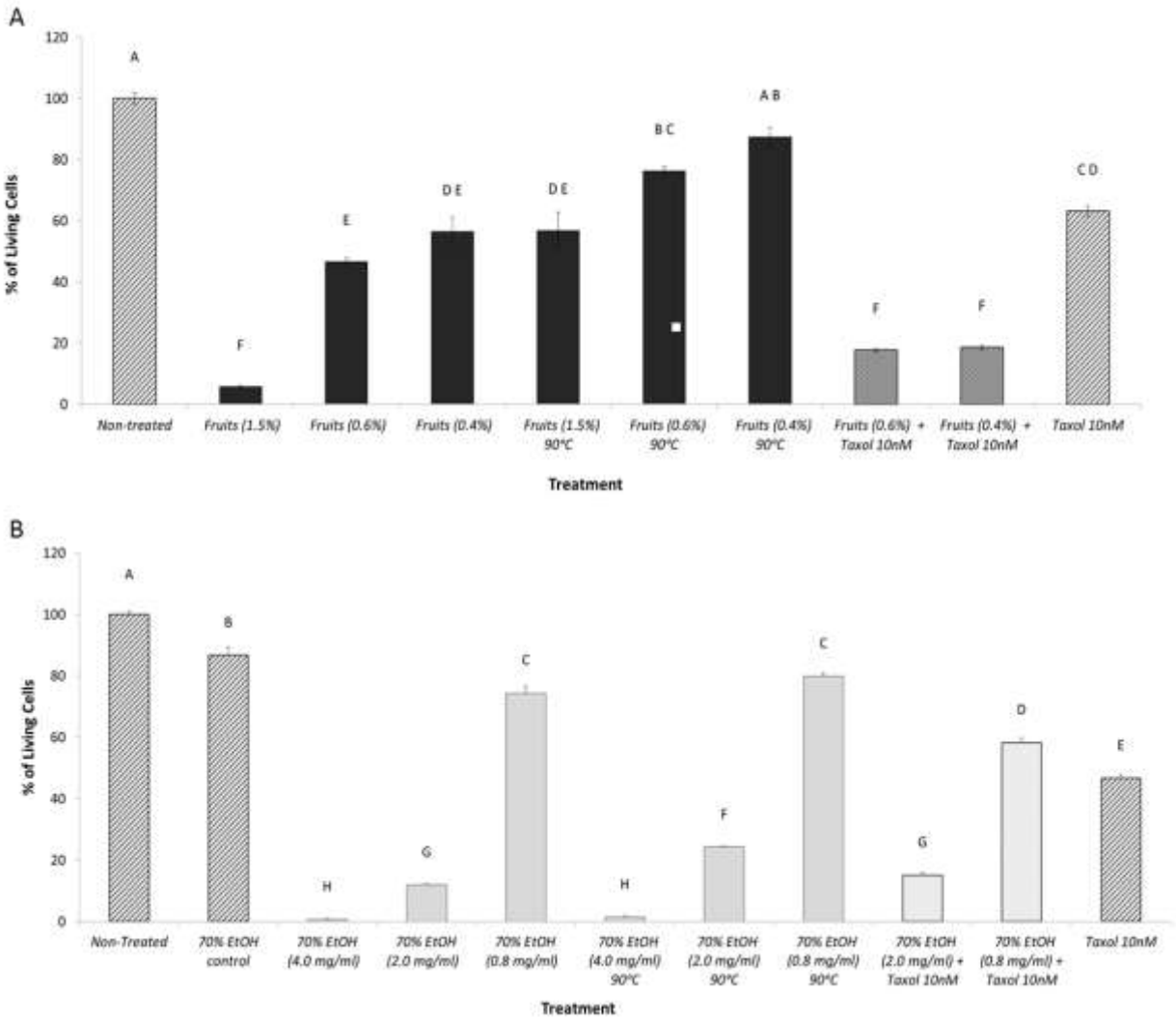

Figure 3. Effect of *E. foeminea* fruit juice (A) and ethanol extract (B) on viability of MDA-MB-231 breast cancer cells. XTT reduction was used to quantify viability. Levels not connected by same letter are significantly different. 70% EtOH, 70% ethanol extract of leaves; Fruit, fruit juice % (v/v); 90°C, heat inactivated extract; Taxol, Paclitaxel.

Table 1. Only sinapyl-related compounds (trans-sinapyl alcohol, trans-sinapyl alcohol derivative and trans-sinapaldehyde derivative, respectively) were present in all the active fractions examined (Table 1); sugar present in all fractions were used as internal standard. Notably, sub-fraction 7.1, inactive for cell viability, did not contain sinapyl-related compounds.

DISCUSSION

The effect of different extracts of *E. foeminea* leaves and fruit juice on cancer and non-cancer cells, and on the cell cytoskeleton was examined. The results based on *in vitro* assays raise concerns that the folkloric use of *E. foeminea* decoction (water extract) does not have a significant effect on cancer cell viability. However, both *E. foeminea* leaf ethanol extract and fruit juice have a significant ability to reduce cell viability. It is possible that the water extraction method of boiling the leaves reduces its activity, and that some heat-labile compounds are being inactivated in the process. Indeed, some of the components in *E. foeminea* fruit juice and to a lesser extent, the ethanol extract, were found to be heat-labile; activity of the ethanol extract was reduced once heated. However, the lack of activity of the *E. foeminea* water extract stands in stark contrast to the recent sporadic reports of improvement in the medical condition of cancer patients following its use. The inefficacy of *E. foeminea* water extract was also reported in Ben-Arye et al. (2016).

The reduction in cancer cell viability by *E. foeminea* ethanol extract and fruit juice was shown on two different

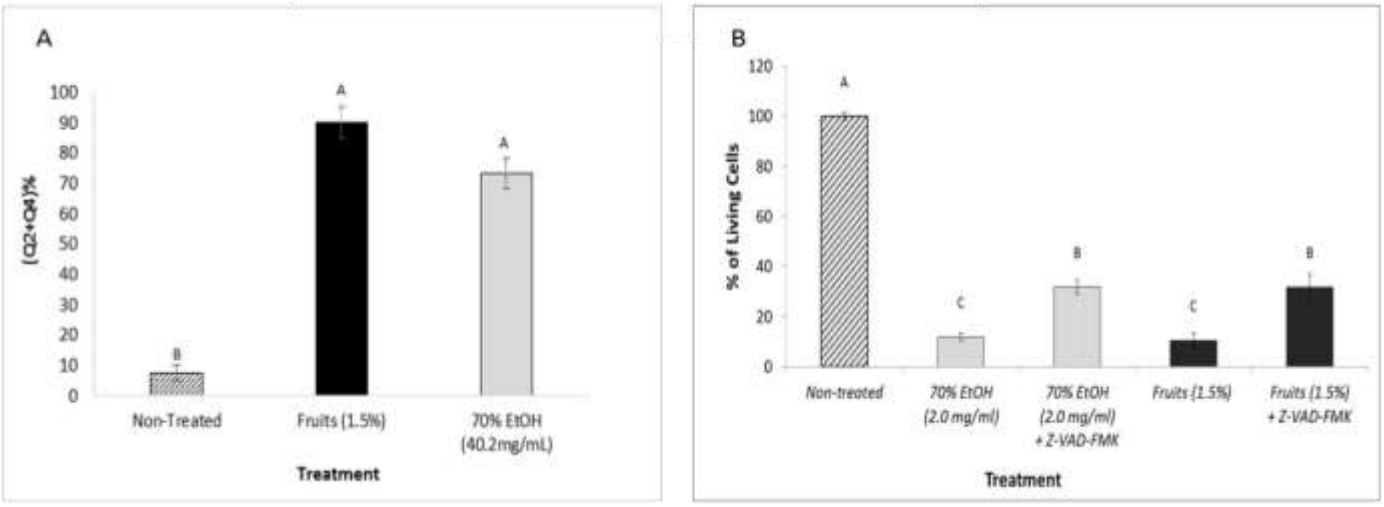

Figure 4. Effect of *E. foeminea* extracts on cell death of MDA-MB-231 breast cancer cells. (A) Results of Annexin V/PI staining assay to assess apoptosis in MDA-MB-231. Cells were then stained using Alexa Fluor® 488 (Life Technologies, Cat # V13241, Eugene, Oregon, USA) or FITC (Invitrogen, Cat # V13242, Eugene, Oregon, USA) solution and propidium iodide (PI) working solution followed by incubation in room temperature for 15 min in the dark. Flow cytometry was performed using GALLIOS flow cytometer. Cells were considered to be apoptotic if they were Annexin V+/PI- (early apoptotic) and Annexin V+/ PI+ (late apoptotic). Live cells were Annexin V-/PI-. Q4, early apoptosis. Q2, late apoptosis. (B) The effect of Z-VAD-FMK (N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone, 100 µM, Abcam, Cat # ab120382, Cambridge, United Kingdom), caspase-3 inhibitor, on MDA-MB-231 cells. Levels not connected by same letter are significantly different. 70% EtOH, 70% ethanol extract of leaves; Fruit, fruit juice % (v/v); Z-VAD-FMK, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone treatment.

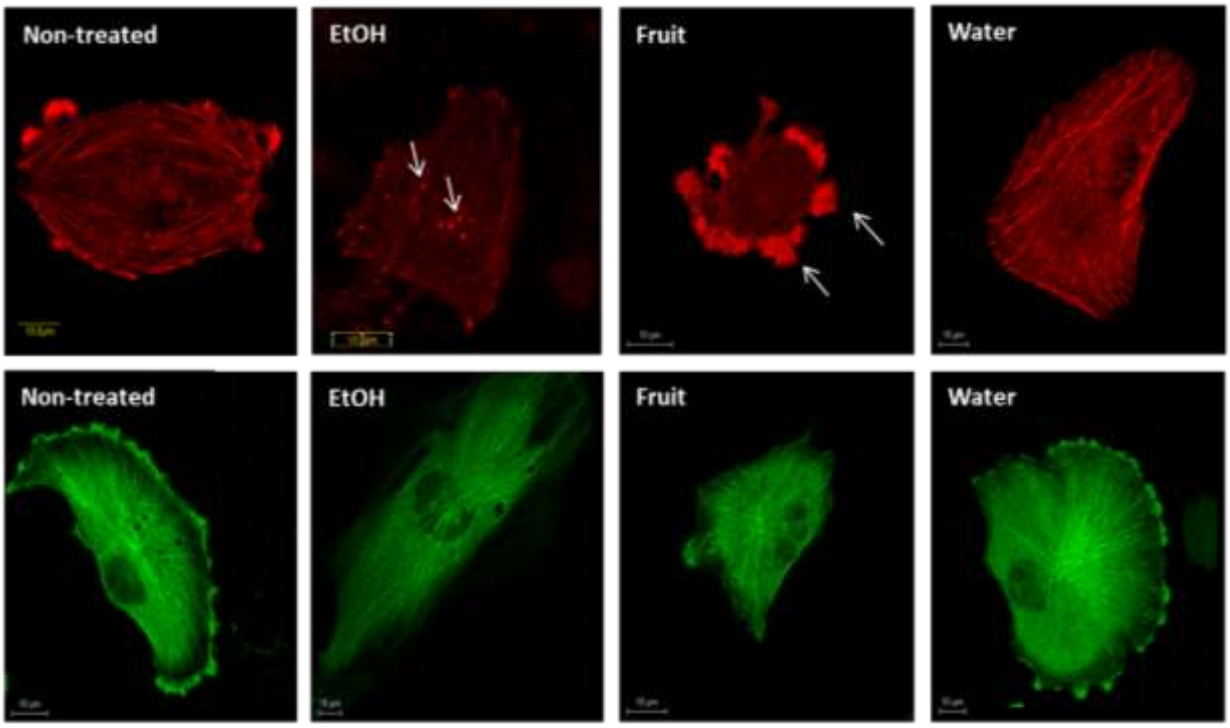

Figure 5. Effects of *E. foeminea* extracts on cell cytoskeleton. Representative fluorescent images of MDA-MB-231 cells transfected with markers for actin (red staining: Life Technologies, Cat # C10502, Eugene, Oregon, USA) and tubulin (green staining: Life Technologies, Cat # C10509, Eugene, Oregon, USA) following treatments with ethanol extract (8 mg/ml extract in the reaction mix), fruit juice (6% v/v extract in the reaction mix), water extract (30% v/v extract in the reaction mix), and non-treated control for 90 min. Images were taken using Leica SP8 laser scanning confocal microscope at 63x magnification.

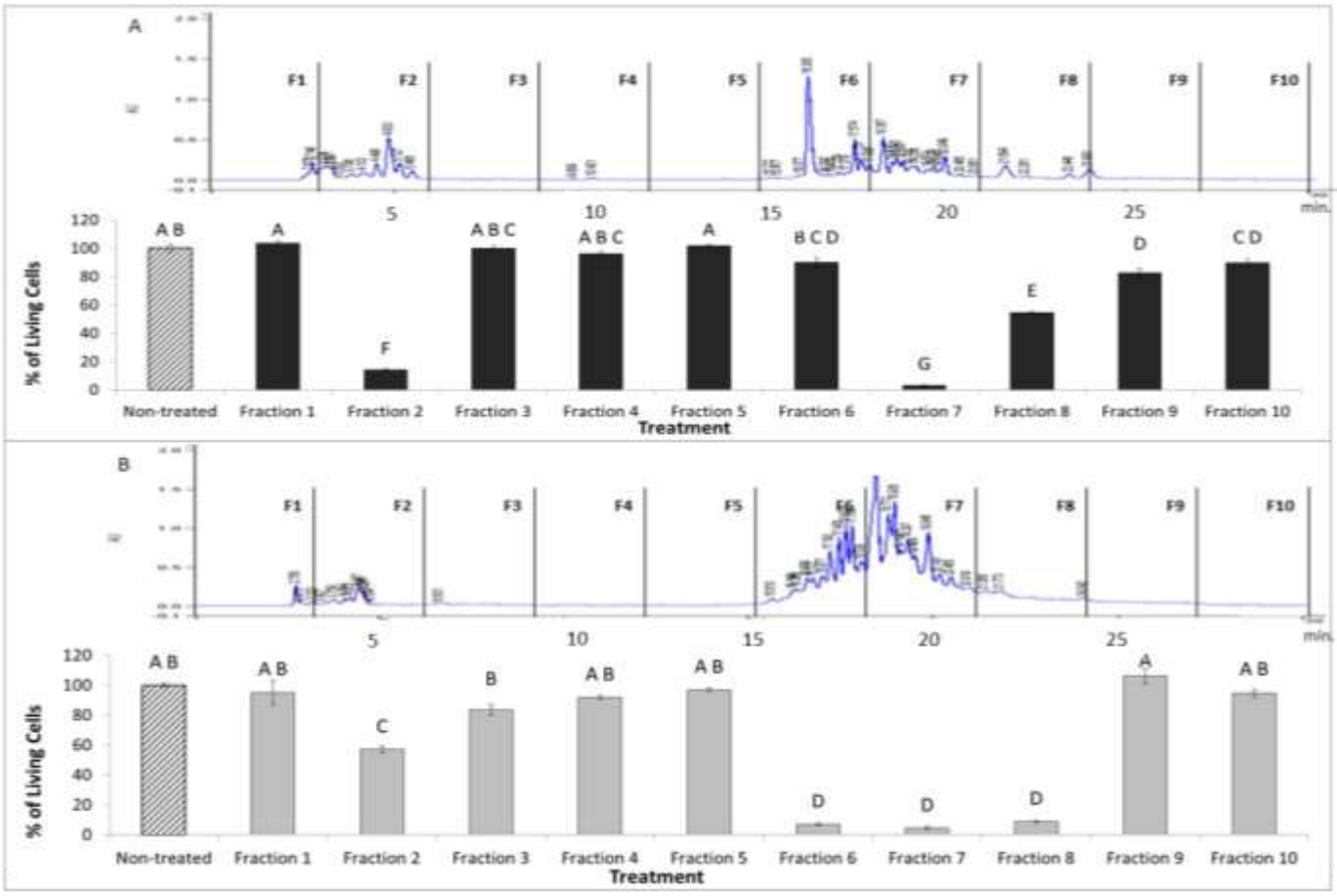

Figure 6. Chemical profiling of *E. foeminea* extracts and activity on cell viability. HPLC chromatogram and XTT of fractions from (A) *E. foeminea* fruit juice and (B) *E. foeminea* 70% ethanol leaves extract obtained from flow gradient with 0.1% acetic acid in water as Solvent A and methanol as Solvent B. The 1 ml min^{-1} of flow started initially with 10% of Solvent B for 10 min and then increased gradually up to 65% from 10 to 40 min, followed by 65% of Solvent B from 40-45 min. Fractions 1-10 represents fractions collected every 3 min. XTT was performed using 3 cycles of HPLC for each fraction (each cycle is 50 µl of extract).

types of cancer cell lines, MDA-MB-231 (breast cancer) and HCT116 (colon cancer), but not on A549 (lung cancer) cells. A somewhat reduced effect was also recorded on non-cancer skin cells (HaCaT). The fact that ethanol extract and fruit juice significantly reduced HaCaT viability may suggest that despite the potential beneficial activity of the *E. foeminea* ethanol extract and fruit juice on cancer cells, they may have adverse cytotoxic effects on non-cancer cells and tissues. The ineffectiveness of all extracts on A549 cell line may result from the insensitivity of this cell line to the cytotoxic compounds in *E. foeminea* extracts. However this needs to be further investigated.

The cytotoxic activity of *E. foeminea* ethanol extract and fruit juice is mediated, at least partially, by induction of apoptosis via a caspase 3-dependent pathway. This cytotoxic activity was enhanced by Taxol, and *vice versa*, that of Taxol was enhanced by the extract and juice, suggesting that they may act together to enhance cancer cell death.

E. foeminea ethanol extracts caused changes in the organization of actin filaments, leading to the formation of invadopodia-like structures. Invadopodia are actin-rich protrusions of the plasma membrane associated with cell migration and metastasis (Yamaguchi, 2012). Part of the activity of invadopodia in invading extracellular membranes is due to localized proteolytic activity of MMPs (Yamaguchi, 2012), involved in tumor metastasis (Foda and Zucker, 2001). In particular, MMP9 was found to be produced mainly by the MDA-MB-231 tumor cells and to significantly contribute to metastatic progression (Mehner et al., 2014). The fact that treatment with the *E. foeminea* ethanol extract leads to a marked increase in MMP9 expression suggests that this extract may indeed induce formation of invadopodia-like structures.

Fruit juice did not lead to the formation of invadopodia-like structures, but rather to the formation of what might be large focal adhesion points. The latter are also found as a result of treatment with Taxol. In human umbilical vein endothelial cells (HUVECs), it was shown that Taxol

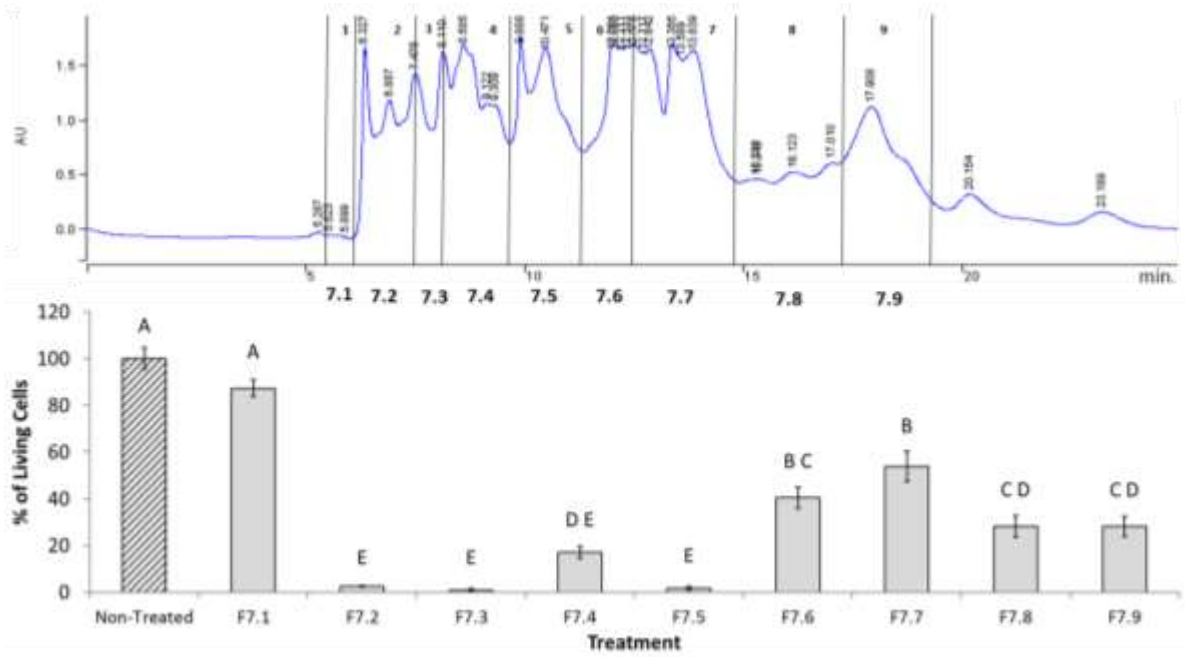

Figure 7. Chemical profiling of fraction 7 of *E. foeminea* ethanol extract and activity on cell viability. HPLC chromatogram and XTT of sub-fractions of fraction 7 of *E. foeminea* ethanol extract obtained from isocratic flow with 0.1% acetic acid in water as Solvent A and methanol as Solvent B. 0.5 ml min^{-1} of flow with constant 40% methanol was used for 25 min. Sub-fractions 7.1 to 7.9 were collected and XTT was performed.

Table 1. Compounds identification and relative amount of sinapyl related compounds in sub-fractions of fraction 7 of *E. foeminea* ethanol extract. Compounds identification for each sub-fraction was obtained using GCMS. *Relative amount of sinapyl related compounds was normalized to D-Allose (sinapyl related compound\D-Allose area). **Relative amount of sinapyl related compounds was normalized to β-Ribose (sinapyl related compound\ β-Ribose area).

Fraction	Identified compounds	Relative amount of sinapyl related compounds*		
		trans-sinapyl alcohol	trans-sinapyl alcohol derivative	trans-sinapaldehyde derivative
F7.1	D-Allose, Xylose	-	-	-
F7.2	β-Ribose, trans-Sinapaldehyde derivative	-	-	1.162**
F7.3	D-Allose, trans-Sinapaldehyde derivative, trans-Sinapyl alcohol derivative	-	2.028	0.57
F7.4	D-Allose, β-Ribose, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, trans-Sinapyl alcohol derivative	-	2.907	-
F7.5	D-Allose, oxalic acid, benzendiol, benzaldehayde, phenol, β-Mannose, hydroxymethoxyphenol, Xylose, L-mannose, β-Ribose, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, C20Ol, trans-sinapyl alcohol, trans-Sinapaldehyde derivative, trans-sinapyl alcohol derivative, ketone	0.294	0.355	0.102
F7.6	D-Allose, trans-sinapaldehyde derivative, trans-sinapyl alcohol derivative	-	0.814	0.253
F7.7	D-Allose, β-mannose, C20Ol, trans-sinapaldehyde derivative, trans-sinapyl alcohol derivative	-	0.462	0.272
F7.8	D-Allose, phenol, hydroxymethoxyphenol, 5-(3-hydroxypropyl)-2,3-dimethoxyphenol, C20Ol, trans-sinapyl alcohol derivative	-	3.499	-

inhibits cell migration, at least in part, by disruption of the regulated formation and turnover of focal adhesions (Kamath et al., 2014). However, despite the effect on actin, and actin's involment in cell migration (Yamaguchi

and Condeelis, 2007), there was no significant effect on cell migration by any of the *E. foeminea* extracts or fruit juice.

Taxol affects tubulin by inhibiting the dynamic instability of microtubules (Schiff et al., 1979; Yvon et al., 1999). However, no changes in tubulin structure could be detected in MDA-MB-231 cells treated with *E. foeminea* extracts suggesting that the effect of the extracts are mainly on actin structures. Taken together, Taxol and *E. foeminea* extracts may affect different components, that is, microtubules and actin, respectively, leading to enhanced aberrations of cell cytoskeleton.

When *E. foeminea* ethanol extract and fruit juice were fractioned, common active fractions and fractions with different activity levels were identified in the two *E. foeminea* extracts, suggesting differences in chemical composition. To further identify active compounds in *E. foeminea*, the active ethanol fraction (Fraction 7) was sub-fractionated and subjected to GCMS analysis, which revealed the presence of several compounds. Only sinapyl-related compounds (trans-sinapyl alcohol, trans-sinapyl alcohol derivative and trans-sinapaldehyde derivative) were present in all active fractions. Interestingly, derivatives of sinapyl alcohol were previously found in *Ligularia nelumbifolia* (Zhao et al., 1994), a plant commonly used in the Chinese medicine for reducing inflammation, for the treatment of coughs, for curing apoplexy and for the treatment of tuberculosis (Zhao et al., 1994). Moreover, it was shown that derivatives of sinapyl alcohol are cytotoxic against several cancer cell lines (Zhao et al., 2002; Zou et al., 2006). It might be that these compounds are responsible for the cytotoxic activity against cancer cells of the *E. foeminea* ethanol extract. However, further research is needed to fully characterize the activity of sinapyl-related compounds, to exclude or include the activity of additional compounds in *E. foeminea* (e.g., those present in fruit juice) and to better understand mode of cytotoxic activity in *E. foeminea*.

Conclusions

The leaf ethanol extract and fruit juice of *E. foeminea* have a significant ability to reduce cancer cell viability. This is mediated at least partially via induction of caspase 3 dependent-cell apoptosis and may be induced by sinapyl-related compounds found in *E. foeminea* ethanol extract. However, cytotoxic activity was low in the water extract widely used in folk medicine. Importantly, the use of this plant for treating cancer may be dangerous, partially due to effects of the extracts on cell cytoskeleton and gene expression associated with cancer cells invasiveness and metastasis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests..

ABBREVIATIONS

Z-VAD-FMK, N-Benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone; **MMP9,** matrix metallopeptidase 9; **DDW,** double distilled water; **EtOH,** ethanol; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase.

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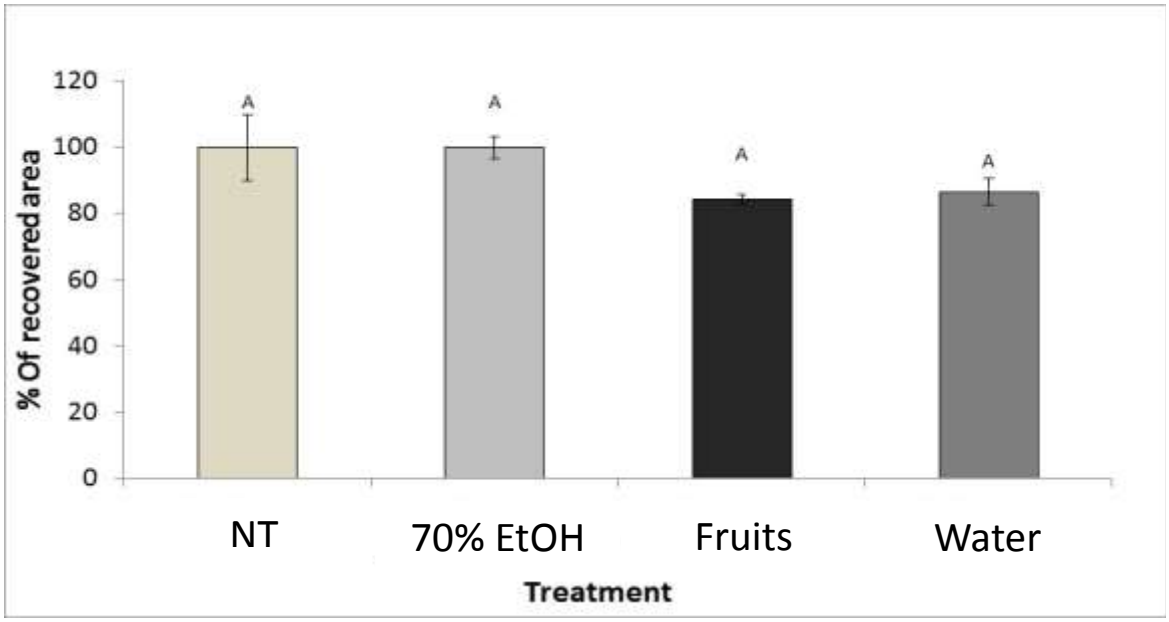
Supplementary Figure 1.



Supplementary Figure 1. Effect of *E. foeminea* ethanol extract and fruit juice on recovered area of confluent monolayers of MDA-MB-231 cancer cell line. Cells were seeded into a 6-well plate at 400,000 cells per well in a volume of 1 ml of growth medium. After 48 h, medium was replaced with treatments including ethanol extract (1 mg/ml extract in the reaction mix), fruit juice (0.3% v/v juice in the reaction mix) and water extract (30% v/v extract in the reaction mix) and cells well incubated at 37°C in a humidified 5% CO_2–95% air atmosphere for 24 h. After incubation, the medium was removed and the cell monolayer was scratched with a 200 µl tip to create a straight line scratch. Three reference points along the scratch are labeled on the bottom of the well, and 1 ml medium containing 1% fetal bovine serum (FBS, to eliminate the possibility of cells filling the scratch by proliferation) was added to each well. Under a phase contrast microscope, images were taken at 0 h. After imaged, the medium was replaced with medium containing the specific treatment and 1% FBS. This procedure was repeated after 24 h. Images of same reference points were taken. The following equation is used to determined cell migration levels: {(0 to 24 h area of treat) / (0 h area of treat)} / {(0 to 24 h area of NT) / (0 h area of NT Avg)} x 100. The values are means ± SE. The statistics was determined by Tukey's HSD test (α =0.05).

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