Functional profiling of *Achillea fragrantissima* (a perennial edible herb) against human cancer cells and potential nutraceutical impact in neutralizing cell proliferation by interfering with VEGF and NF-κB signaling pathways

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Abstract

Chemoprevention with alternative approaches is emerging as a significant component of therapeutic regimen for the management of various diseases in human population including cancer. The concept of personalized nutrition is attracting considerable interest as an effective and affordable strategy in the prevention of chronic diseases. It is acknowledged that diet-derived agents or non-dietary natural products are not only the source of traditional medicines but also the lead compounds for currently used pharmaceuticals with excellent efficacy against a number of human diseases. *Achillea* species (Asteraceae) are considered as functional foods, which are important constituents of traditional medicine and commonly consumed as herbal tea or food additives worldwide. The studies presented herein demonstrate the effects of the hydro-methanolic extract of *A. fragrantissima* against a panel of cancer cells that include breast cancer (MDA-MB-231,MCF-7,SKBR3), pancreatic cancer (BxPC-3, MiaPaCa-2), prostate cancer (LNCaP,C4-2B,PC-3), and lung cancer (A549). The experimental results presented in the study show that the extract, which is rich in structurally diverse phytochemicals, effectively inhibits the cell growth and induces apoptotic cell death in human cancer cells. The treatment of the cancer cells with the extract resulted in a progressive decrease in cell migration and invasiveness, demonstrating an effective anti-metastatic activity. The mechanism by which the extract exerts its effects against cancer cells potentially engages NF-κB signaling and downregulation of its target cytokines such as VEGF. The study provides evidence that partially support the importance of functional foods and highlights their significance in disease prevention.

Keywords: cancer; cell signaling; chemoprevention; functional food; pharmaceuticals

Introduction

The International Agency for Research on Cancer in its recent report has shown a marked increase in the world-wide cancer burden from 14 million annually in 2012 to 18.1 million in 2018 (IARC-GLOBOCAN, 2018). Over the same period, the rate of cancer-related mortality increased from the estimated figure of 8.2 million to

9.6 million annually. It has been realized that in recent years, there has been a substantial rise in the interest towards alternative approaches for the treatment of chronic disorders and the demand for adopting such practices is growing rapidly (Tangkiatkumjai *et al*., 2020). According to the World Health Organization, a large percentage of people (≈80%) from the low- and middle-income countries depend on traditional medicine for their health care management (WHO, 2002). Moreover, 25% of drugs prescribed for various ailments across the globe are plant derivatives (Rates, 2001). There are evidences in clinical practice to suggest that the phytochemicals derived from natural sources have contributed in a significant way in drug discovery, disease prevention, and human health (Newman and Cragg, 2020). Over the years, several phytochemicals (such as phenolic compounds), including resveratrol, curcumin, quercetin, genistein, tannic acid, caffeic acid, or bioactive extracts of medicinal plants, have shown diverse chemopreventive properties (Del-Toro-Sánchez *et al*., 2021; Tapia-Hernández *et al*., 2018; Ullah *et al*., 2014). These observations encourage the utilization and mapping of current knowledge with the traditional practices incorporated in the ancient renaissance, which strongly engages the belief in the practice of disease management through natural products and promotes dietary ingredients of food in various ethnic cultures as therapeutically functional entities (Fernandes et al., 2022; Santini, 2022; Ullah et al., 2022; Zhou *et al*., 2020). Moreover, such preventive strategies based on components of regular human consumption have reasonable safety index and the plausibility of undesired effects are either none or least. The concept of personalized nutrition is attracting considerable interest as an effective strategy in the prevention of chronic diseases. The wide variety of components within the dietary and non-dietary plant sources may contribute to an extent in mitigating the impediments associated with chemo- and targeted therapies, while displaying strong anticancer activities. The genera *Achillea* (Asteraceae) constitute over 115 species, which are widely distributed perennial herbs in European countries and in the Arabian Peninsula (Nemeth and Bernath, 2008). Interestingly, the nomenclature Achillea is believed to be derived from Achilles (Greek warrior), who used this plant species for treating soldiers injured during the course of the famous Trojan War. The *Achillea* species are extensively used in folk or traditional medicine in the treatment or prevention of several diseases (Han and Bulut, 2015). *Achillea millefolium* has been reported to have beneficial effects in neurodegenerative diseases like Alzheimer's (Elmann *et al*., 2011), whereas *Achillea eriophora* and *Achillea biebersteinii* provide protection against oxidation, cytotoxicity, and DNA damage (Varasteh-Kojourian *et al*., 2017). *A. fragrantissima* is a herbal species with documented pharmacological properties that include anti-inflammatory, antidiabetic, and anti-cancer properties, along with some evidence of neuroprotective effects (Abdel Fattah *et al*., 2018; Choucry, 2017). Studies on toxicity profiling have shown neither acute nor sub-chronic toxicity in animal models with the different extracts of *A. fragrantissima* (Mandour *et al*., 2013). The use of herbal teas prepared from some *Achillea* species in traditional therapies also demonstrates the safety of the formulations in human subjects (Bali *et al*., 2015). The present study investigates the consequences of hydro-methanolic extract of *A. fragrantissima* against a panel of cancer cells that include breast cancer (MDA-MB-231,MCF-7,SKBR3), pancreatic cancer (BxPC-3, MiaPaCa-2), lung cancer (A549), and prostate cancer (LNCaP,C4-2B,PC-3). Our study further highlights the potential of the extract in the inhibition of cancer cell proliferation, induction of apoptosis, and modulation of growth mediators such as NF-κB and vascular endothelial growth factor (VEGF). It has been shown that targeting of NF-κB activation and VEGF, and their subsequent inhibition is an important action mechanism of certain anti-cancer therapy (Li *et al*., 2013).

Materials and Methods

Materials, chemicals, and reagents

The aerial parts of *Achillea fragrantissima* were collected in the spring season from its natural habitats in Deesa Valley in the north of Saudi Arabia. The authentication of the plant material was performed by the Department of Botany, Faculty of Science, University of Tabuk (RH37). Cancer cell lines PC3, LNCaP, C42B, MDA-MB-231, MCF-7, SKBR3, MiaPaCa-2, BxPC-3, and A549 were procured from ATCC (Manassas, VA, USA). RPMI 1640 (Invitrogen, Carlsbad, CA, USA) was used for PC3, LNCaP, C42B, and SKBR3 cells, whereas DMEM (Invitrogen, Carlsbad, CA, USA) was used for MDA-MB-231, MCF-7, BxPC-3, MiaPaCa-2, and A549 cell lines. The supplementation of the media with 10% fetal bovine serum was followed with the addition of antibiotics penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were cultured in a 5% CO_2-humidified atmosphere at 37°C. Stock solution of the extract was made by dissolving in DMSO, and fresh stocks were made for every individual assay. NF-κB p65 Transcription Factor assay kit was purchased from Abcam, USA (Catalog #ab133112) and VEGF assay ELISA kit was from R&D Systems, Inc. (USA). Pyrogallol, tannic acid, and quercetin were obtained from Bayer Pharma, India. Organic solvents used were of HPLC grade from India (Rankem). All other chemicals were also of analytical grade.

Extraction process

The extraction was carried out using the protocol as reported earlier (Ullah *et al*., 2015), with some minor modifications. The aerial parts of *A. fragrantissima* were immediately rinsed with cold water and dried under shade. In general, hot water rinse is considered better to remove surface dirt and residues. However, based on our observations, it is believed that in the case of plant materials which are intended to be stored for longer periods, it is preferable to use distilled cold water for a quick rinsing since it is better in conserving the content while hot water accelerates the decaying process. We can also find in literature the use of tap water for rinsing which is also acceptable provided there is no risk of contamination. The samples were shade dried as shade-drying has a higher capacity to preserve the constituent phytochemicals/nutrients and bioactivities compared to sun drying, whereas other methods such as oven-drying might be preferable in the industrial setup (Yuan *et al*., 2015). It should be noted that phytochemical constituents are heat sensitive and result in lower yield at high temperature drying (Al-Juhaimi *et al*., 2018; Yuan *et al*., 2015). Dried plant materials with moisture content of 10–14% are considered suitable for preventing undesirable microbial growth, limiting enzymatic degradation, and promoting higher shelf-life (Poos and Varju, 2017). The moisture content of the dried material was determined as described earlier (Alara *et al*., 2018) and was found to be 12.8% on day 15, after which the material was further processed. Shade-dried plant material was crushed and further ground to fine powder. Dry leaves' powder (250 g) was soaked in 2 L of methanol (80%) in a flask placed at 40°C in water bath for 24 h with continuous shaking. The extracted mixture was then filtered through double-layered clean cheesecloth and double-layered Whatman paper (Sigma-Aldrich Co., St. Louis, MO, USA). The concentrated filtrate (under reduced pressure at 35°C using a BuchirotavaporR-210 (Flawil, Switzerland) was placed under vacuum at −30°C for 3–4 days to yield solid/thick pastes. The residual material was weighed to be 50 g giving a total yield of 20% (w/w).

Phytochemical analysis

The hydro-methanolic extract was subjected to analysis for the presence of different kinds of phytochemicals with diverse structure such as phenolics, flavonoids, saponins, tannins, and alkaloids (Ullah *et al*., 2015). Total phenolic content was quantitatively determined using pyrogallol as a reference and tannin was determined using tannic acid as a standard. The gravimetric estimation described by Harborne was used for analyzing the contents of alkaloids and saponins, whereas the flavonoid content was estimated as quercetin equivalents.

Cell growth inhibition studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

Cells seeded in 96-well culture plates were left for an overnight incubation. Following incubation, fresh medium was added containing DMSO (vehicle control) or extract in concentrations as indicated for individual experiments. At the end of 4 days of incubation, each well was supplemented with 25 µl of MTT solution (5 mg/mL in PBS) and further incubated for 2 h at 37°C. After aspirating the supernatant, the MTT formazan, formed by metabolically viable cells, was dissolved in DMSO (100 µl) by mixing for 30 min on a gyratory shaker. The absorbance (595) nm was recorded on a microplate reader (Ultra Multifunctional Microplate Reader-TECAN, Durham, NC, USA) (Ahmad et al., 2010). Each treatment had eight replicate wells and in each reaction mixture, the amount of DMSO never exceeded 0.1%. The experiments were replicated at a minimum of three times.

Homogeneous caspase-3/7 assay for apoptosis

The apoptosis detection assay was performed, as reported previously (Ahmad *et al*., 2009), using Caspase-3/7 homogeneous assay a kit (Promega (Madison, WI). Different concentrations of extracts or DMSO (vehicle control) were used to treat the cells for 96 h. At the end of the treatment, Apo-ONE1 caspase-3/7 reagent (100 µl) was added; plates were shaken for 2 min and further incubated at room temperature for 3 h. The fluorescence of the test samples was then measured using ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 485/530 nm.

Soft agar colonization assay

A 24-well plate that contained 0.3% (w/v) top agar layered over a basal layer of 0.7% (w/v) agar (with culture medium and the supplements) was used to seed cells (3×10^4) in 0.5 mL of the culture medium. The culture was supplemented with different concentrations of extracts or the DMSO during the time of seeding. After appropriate culture time (22 days), colonies (>50 cells) were counted (Ahmad *et al*., 2011). Quadruplicate experiments were carried out and the results presented are representative of three independent observations.

Invasion assay

The study for cell invasion potential was carried out using 24-well transwell permeable supports with 8-mm pores (Corning, USA) that are coated with growth factor–reduced Matrigel (BD Biosciences, USA). The assay

was procedure executed according to the instruction guide provided by the vendor. Initially, the cells were treated with trypsin and suspended in serum-free medium. These cells were then seeded into the transwell inserts. The bottom wells were used to load complete media. Following 24 h, cell staining was carried out with calcein AM-4 mg/mL in PBS at 37°C for 1 h (Invitrogen, USA). The cells were then detached from inserts by treatment with trypsin. The fluorescence was recorded for the cells that invaded through matrigel with ULTRA Multifunctional Microplate Reader (TECAN). Arbitrary fluorescence of control (no treatment) groups was set as 1.0 and the relative fluorescence under different treatments was reported.

NF-κB inhibition assay

Assay kit "NF-κB p65 Transcription Factor Assay Kit" was obtained from Abcam (Catalog # ab133112). The experiment was performed in stepwise protocol mentioned in the instruction guide with a minor alteration that cells were exposed to PMA (15 ng/mL) for 1 h for the induction of NF-κB before the addition of the indicated doses of extract to test for their ability to inhibit NF-κB activation. PMA which has been taken as a positive control has been shown to activate NF-κB in a dose-dependent manner (Hellweg *et al*., 2006). The absorbance is proportional to the NF-κB detected in the assay and represents a semi-quantitative analysis to measure the NF-κB modulatory properties of a compound or formulation.

Elisa assay for VEGF secretion

The assay was performed by initially seeding a definite number of cells in six-well plates, which were left for an overnight incubation. Following the incubation, cells were treated with indicated doses of extract for 36 h, after which the culture media was collected and centrifuged (800 g × 5 min) at 4°C to separate the cell debris. The supernatant was aspirated carefully without disturbing the cell debris, and was used immediately for VEGF assay using an ELISA kit (R&D Systems, USA) following the manufacturer's instructions. The cells attached to the plate were subsequently treated with trypsin, re-suspended, and counted using a hemocytometer.

Statistical analysis

The experimental data were obtained for three separate sets, and each set has a triplicate measurement (or otherwise as indicated in the legends). The data are reported as the mean values ± SE. Statistical analysis was performed for analysis of variance (ANOVA) which was followed by F-test with SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA). For all experimental data, the values of p, which were 0.05 or less, were considered significant. For each data set, the individual P-values are reported in the figures, as appropriate.

Results and Discussion

Extract of A. fragrantissima evince the presence of structurally diverse plant-derived bioactive molecules

The extract of *A. fragrantissima* when subjected to quantitative analysis for the phytochemical contents exhibited the presence of major secondary metabolites of plant origin, which have been widely reported in literature for their pharmacological properties (Velu *et al*., 2018). The estimation was made for different kinds of molecules such as flavonoids, tannins, phenolics, saponins, and alkaloids, which are present in varying concentrations in the hydro-alcoholic extract, as shown in Figure 1. Specifically known phytochemicals (Table 1) present in this plant include sesquiterpene lactone, Achillolide A, 3,5,4'-trihydroxy-6,7,3'-trimethoxyflavone, cirsiliol, cirsimaritin, and a number of hydrocarbon compounds which have been reported to possess pharmacological properties including anticancer effects (Barda *et al*., 2021; Bartolotti *et al*., 2018; Patocka and Navratilova, 2019). Studies have shown that various secondary metabolites of plant origin have the ability to inhibit the process of carcinogenesis in preclinical and clinical models (Ullah *et al*., 2014).

Phytochemicals such as tannins have been reported to induce cell cycle arrest, cell death, and inhibit the proliferation of various cancer cells including breast, prostate, liver, and colon cancer by interfering with signaling cascades involved in cell survival and apoptotic pathways (Baer-Dubowska *et al*., 2020). The condensed tannins such as proanthocyanidins are major constituents of fruits such

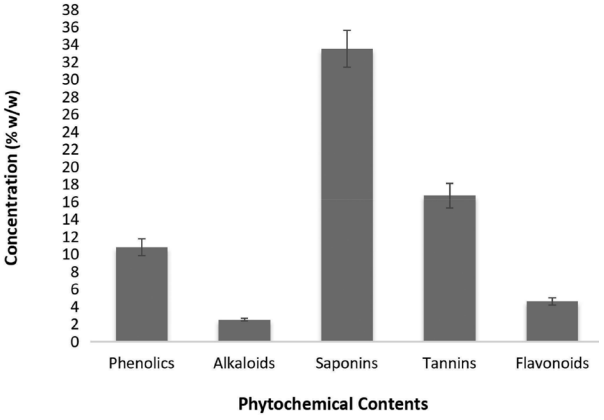
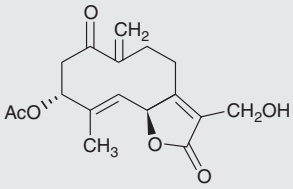
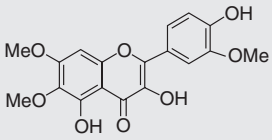
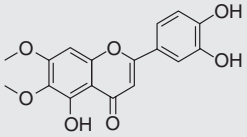
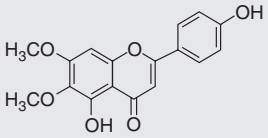


Figure 1. Bioactive molecules with diverse structures and pharmacological relevance form the phytochemical constituents of *A. fragrantissima* extract.

Table 1. Chemical structures of bioactive phytochemicals.

Phytochemicals	Chemical Structures
Achillolide A	
3.5.4'-trihydroxy-6.7.3'-trimethoxyflavone	
Cirsiliol	
Cirsimaritin	

as apple; these are also found to be major constituents of beverages such as coffee and tea. The alkaloids, which are nitrogen containing basic compounds, possess considerable structural diversity and contribute to varied effects on human physiology. Alkaloids derived from herbal sources have shown significant anti-proliferative and anti-cancer effects on different cancers both *in vitro* and *in vivo*, with some emerging as highly efficacious anti-cancer drugs such as vinblastine, vinorelbine, vincristine, and vindesine (Mondal *et al*., 2019). Studies have also reported the cytotoxic properties of certain saponins against cancer cells, with the treatments showing saponins to be either comparable or more effective than doxorubicin and paclitaxel which are well-known anticancer drugs (Sobolewska *et al*., 2020). Plant-derived phenolic compounds such as flavonoids possess multiple mechanisms of action against cancer. These mechanisms include generation of ROS, modulation of phase I and II xenobiotic metabolizing enzymes, influencing epigenetic modifications and the dynamic state of chromatin, anti-inflammatory properties, mediating pro-apoptotic and anti-proliferative effects by interfering with NF-κB, Wnt/β-catenin, and MAPK pathways, and modulating EMT pathway in cancer metastasis and invasion (Avila-Carrasco *et al*., 2019; Farhan and Rizvi, 2022; Lewandowska *et al*., 2016; Ullah et al., 2016, 2022). Relatively higher intake of fruits and vegetables, which are rich sources of flavonoids, has been epidemiologically linked to a lower risk of cancer incidence (Wang *et al*., 2015). These secondary metabolites have important roles in plants including defense by phytoalexins against undesired pathological and environmental stress. Such molecules when they interact with animal or human cells can interfere with cellular signaling through different mechanisms that could be tailored for pharmacological activities. It is thus presumed that the pharmacological properties associated with *A. fragrantissima,* including the cytotoxicity against cancer cells, might be ascribed to the presence of an array of diverse bioactive secondary metabolites. It might be mentioned that either one phytochemical constituent can induce such effects or in case of an extract or whole food, the possibility of synergistic mechanism of action is consequentially significant.

Inhibition of cell proliferation in cancer cells treated with incrementing concentrations of *A. fragrantissima* extract

The anti-proliferative properties of the extract were investigated by exposing cancer cells derived from different cell lines including prostate (PC3, LNCaP, C42B), breast (MDA-MB-231, MCF-7, SKBR3), pancreatic (MiaPaCa-2, BxPC-3), and lung (A549) cancer cells to various doses of the extract for 96 h to examine the effect of the treatment on cellular proliferation. These cell lines are often used as representative prototypes for examining the cytotoxic properties of a compound or a drug as an *in vitro* cancer model. Our results (Figure 2) based on MTT assay show that the extract causes a dose-dependent inhibition of the cell proliferation in the tested cancer cell lines ($P < 0.01/P < 0.05$).

The effect of the incremental doses shows the increase in the tested concentrations of the extract result in a corresponding decrease in the cell viability. As indicated by the IC_{50} values reported in Table 2, the extract showed most effective inhibitory effects against SKBR3, LNCaP, C4-2B, and BxPC-3 cells with an IC_{50} value of 44.2 µg/mL, 45 µg/mL, 51.8 µg/mL, and 60.2 µg/mL, respectively. These results show significant cytotoxic effects against all the tested cancer cell lines, which provide evidence of a cancer-specific action of the extract. As reported in the figure, at certain concentrations of the extract, 80–90% inhibition of cell growth was observed in specific cancer cell lines. Studies on other species of the plant have also shown anti-proliferative effects such as the extract obtained from the flowers of *Achillea flipendulina* has been reported to possess cytotoxic properties against breast (MCF-7) and liver (Hep-G2) cancer cells (Asghari *et al*., 2020). Studies on plant extracts with saponins rich fractions have been recently shown to exhibit cytotoxic activity with significantly lower IC_{50} values of ˜4 µg/mL and ˜5 µg/mL against MDA-MB-231 and MCF-7 cancer cell lines, respectively (Amrati *et al*., 2020). These results imply that *A. fragrantissima* extract has significant growth inhibitory and antiproliferative activity against

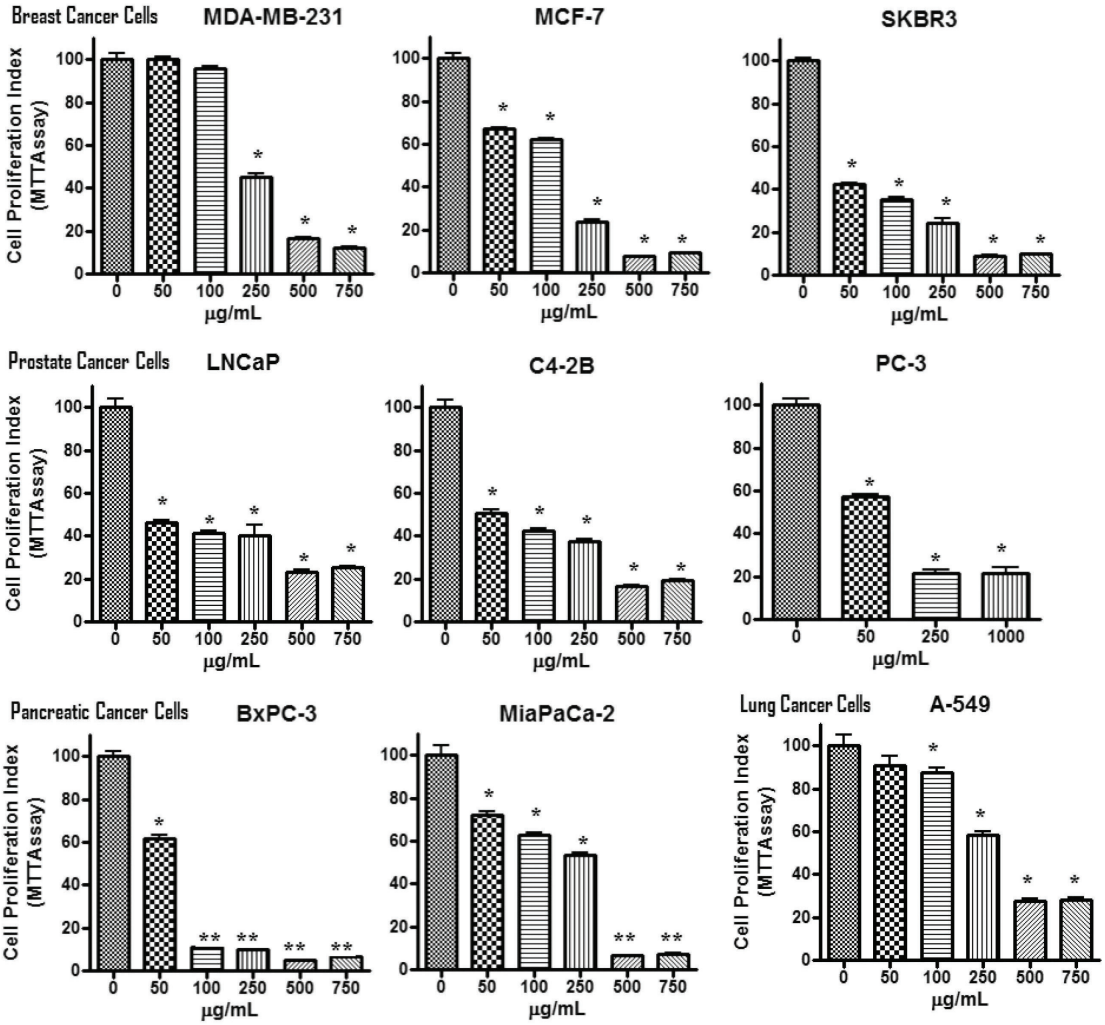


Figure 2. Inhibition of cell growth in cancer cell lines by the indicated doses of *A. fragrantissima* extract (96 h) expressed as percentage of control ± S.E. of triplicate determinations from three independent experiments (P < 0.01/*P < 0.05).**

Table 2. Antiproliferative effects of hydro-alcoholic extract of *A. fragrantissima* against cancer cells.

Cancer cell lines		IC50 µg/mL (96 h)
Breast	MDA-MB-231	236.6 ± 5.6
	MCF-7	150.2 ± 3.4
	SKBR3	44.2 ± 0.8
Prostate	LNCaP	45.0 ± 0.9
	C4-2B	51.8 ± 0.7
	PC3	80.0 ± 1.1
Pancreatic	MiaPaCa-2	280.1 ± 8.2
	BxPC3	60.2 ± 1.0
Lung	A-549	318 ± 9.6

different cancer cells, however with varying sensitivity as observed in dose-response curves for different types of cancer. Different cancer cell types possess variations in genetic abrasions and specific alterations in cellular and metabolic pathways, which influence the sensitivity towards a cytotoxic agent.

A. fragrantissima extract causes induction of caspase-dependent apoptotic cell death in cancer cells

The inhibitory potential of anti-cancer agents against cell growth in cancer cells is known to be associated with cell death pathways, particularly following the induction of apoptosis (Ahmad *et al.*, 2010). Therefore, our study further explored the ability of the extract to induce apoptotic cell death in various cancer cell lines, which were also sensitive to the antiproliferative action of the extract reported in the MTT assay. The induction of apoptosis mediated by the extract in the tested cells was characterized by the assessment of the activation of caspases, which showed noticeable caspases-3 and caspase -7 expressions in these cells. We performed the caspase-3/7 homogeneous assay, which quantifies the rate

of apoptosis based on the levels of active caspase-3 and/or caspase-7 by fluorescence activity. Results from this assay (Figure 3) suggested a dose-dependent progressive induction of apoptosis by the extract in cancer cells, as evidenced by enhanced fluorescence activity that is redolent of the presence of active caspase-3/7. Thus, the presence of activated caspases in the treated cells is suggestive of the induction of caspase-dependent apoptosis as these enzymes are involved in the effector phase of apoptosis. Several studies have shown such apoptosis induction by natural products in cancer cells such as lung cancer (NCI-H460/SK-MES-1) by pterostilbene (Schneider *et al*., 2010) and breast cancer (MCF-7/MDA-MB-231) by vernodalin (Looi *et al*., 2013). A series of evidence in literature suggest that various anti-cancer drugs and components of plant extract causes induction of apoptosis in a range of cancer cells (Lin *et al*., 2011; Stepczynska *et al*., 2001). It is considered a hallmark characteristic of cancer cells to evolve mechanisms to evade senescence or apoptosis in order to survive, and agents that can interfere with this evasive cancer cell signaling can tailor the cells to death similar to normal phenotype. There are components of human diets which have been shown to cause apoptosis in cancer cells (Lin *et al*., 2011) and these might contribute in the maintenance of homeostatic state wherein undifferentiated cells of precancerous origins or cancer cells would be coerced to undergo cell death.

A. fragrantissima extract inhibits colonization and retards invasiveness in cancer cells in vitro

Anchorage-independent clonogenic assay is an effective *in vitro* tumorigenicity study model that allows to measure the potential of a single cell to form a multicellular colony in a three-dimensional environment without an adhesion matrix. The study is entrancing as it determines the reproductive ability of a cell when treated with a cytotoxic agent and reflects the *in situ* microenvironment wherein the cancer cells are capable of detaching from the cell matrix for metastatic mobility and still survive by evading anoikis. As shown in Figure 4, treatment of multiple cancer cell lines representing breast, prostate, pancreatic, and lung cancer with the extract demonstrates a progressive decline in colony formation in a dose-dependent manner which was estimated based on the number of colonies formed when compared to the untreated control. Furthermore, these results were

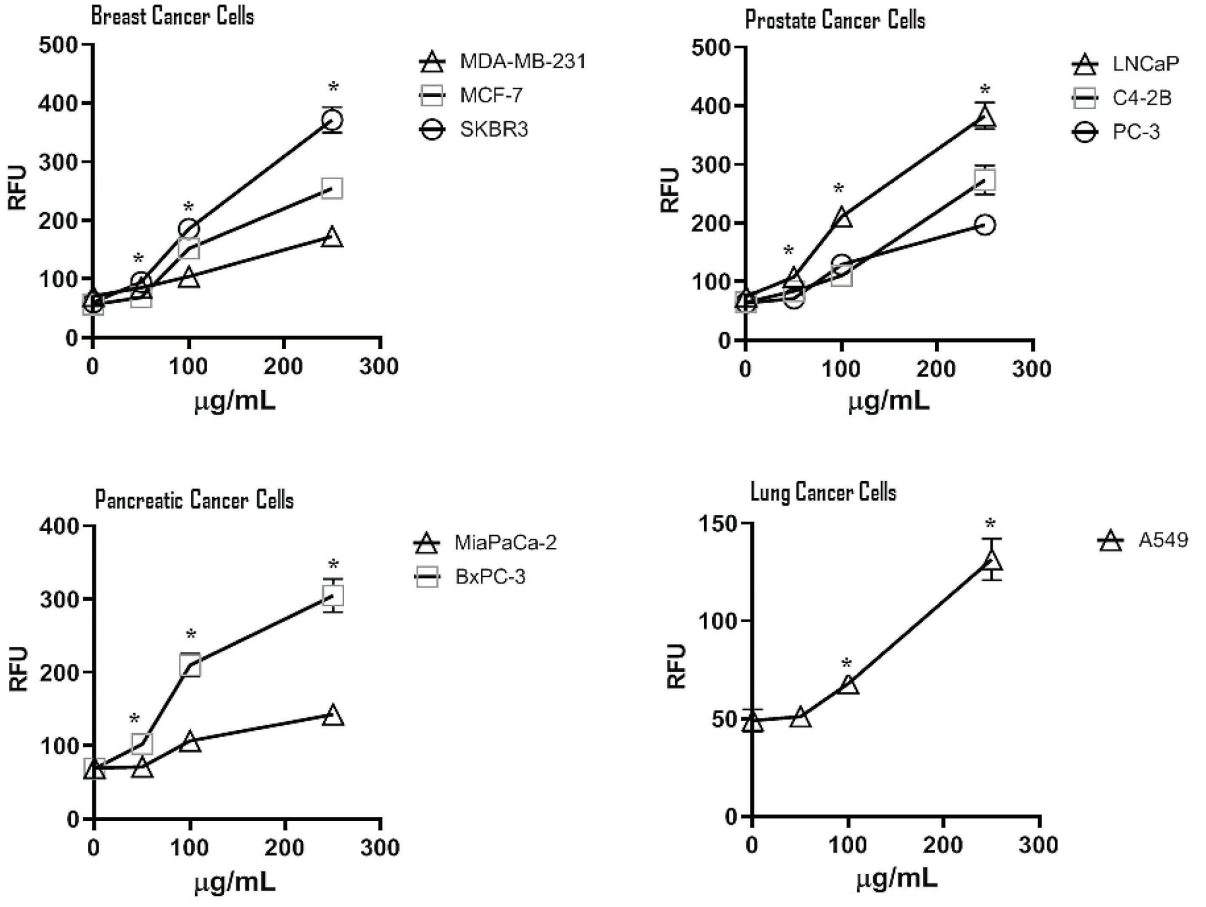


Figure 3. Apoptotic cell death in cancer cells treated with increasing concentrations of *A. fragrantissima* extract for 96 h (*P < 0.05).

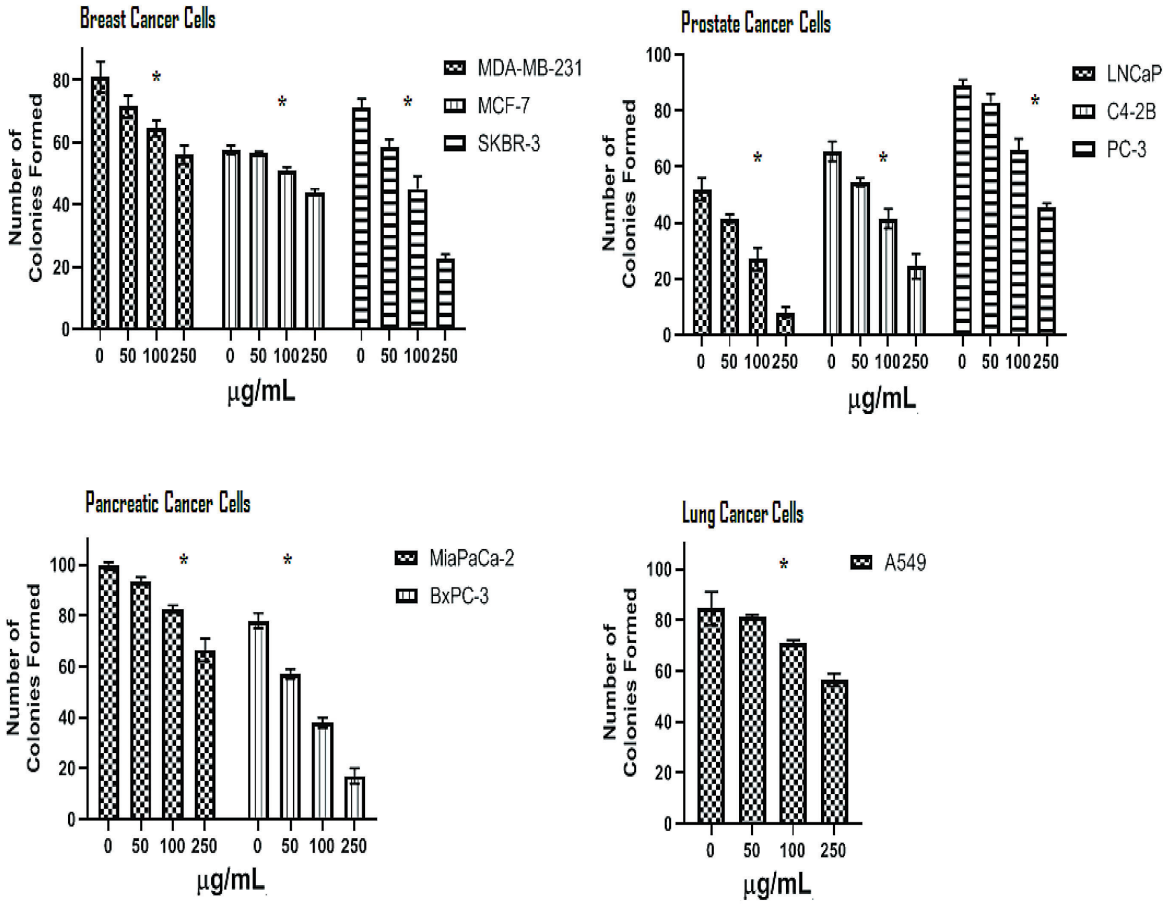


Figure 4. Inhibition of anchorage-independent colonization in cancer cells treated with indicated doses of *A. fragrantissima* extract (P < 0.01).**

complemented with the invasion assay, which reflects the invasive or metastatic potential of cancer cells. It was observed from the graph showing the relative invasive potential of treated cells compared to the untreated control (Figure 5) that the invasiveness, which is measured by the ability to penetrate through the Matrigel-coated membrane significantly declined for treated cells. Consequently, fluorescence values obtained from the migrated cancer cells which were treated compared to control decreased significantly, as the extract retards the metastatic potentiality in treated cells. Cancer metastasis is characterized by an enhanced motility of cancer cells, the ability of the cells to detach from the cell matrix and evade cell death on lacking the adhesion signals leading to invasion and survival in circulation, extravasation, and finally the ability to colonize as tumors at distant sites (Tungsukruthai *et al*., 2018). Several studies investigating the pharmacology of natural product derivatives including phytochemicals have shown promising anti-metastatic activities by vanquishing principal molecular events and sensitizing cancer cells to anoikis (Powan *et al*., 2013). These compounds have been demonstrated to interfere effectively with the metastatic dimensions, such as cell-matrix adhesion, tissue barrier degeneration, epithelial-mesenchymal transition, migration, invasion, and vascularization with both *in vitro* and *in vivo* studies showing promising anti-metastatic outcome (Weng and Yen, 2012; Yen *et al*., 2018).

A. fragrantissima extract inhibits the intracellular targets of cellular proliferation -NF-κB and VEGF

Nuclear factor-κB is a major transcription factor that regulates cellular behavior and outcome associated with immunogenic responses such as inflammation and influences the expression of several genes in cancer cells that are important for proliferation, survival, and invasion (Xia *et al*., 2014). As shown in Figure 6A, the PMA-induced NF-κB activation in prostate, breast, and pancreatic cancer cells were effectively neutralized by the extract. Thus, inhibition of NF-κB by the extract provides an understanding of the mechanistic targets of its anticancer activity.

Moreover, tumor vascularization or angiogenesis is one of the pathological hallmarks of cancer growth and metastasis (Xie *et al*., 2010). It was reported earlier that inhibition of NF-κB signaling retards cancer growth and

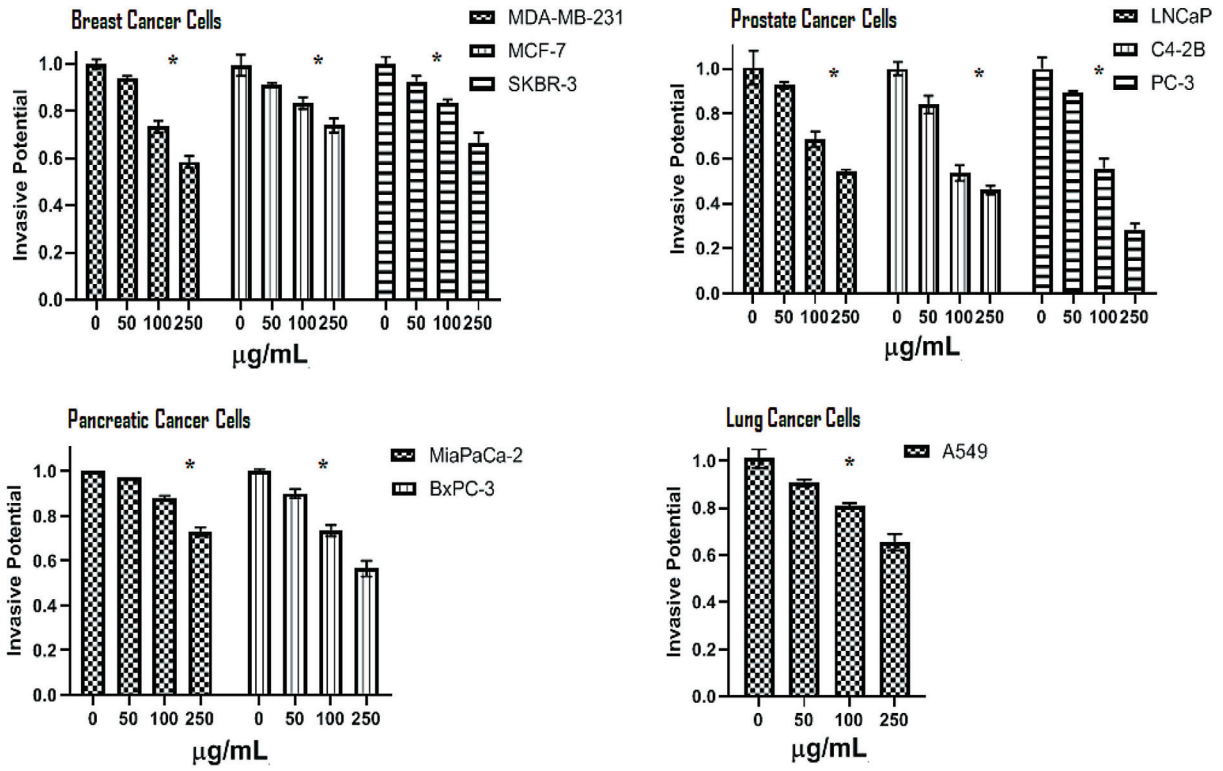


Figure 5. Inhibition of invasive potential reflecting the ability of cells to infiltrate through the Matrigel-coated membrane by varying doses of *A. fragrantissima* extract in treated cancer cells (*P < 0.05).

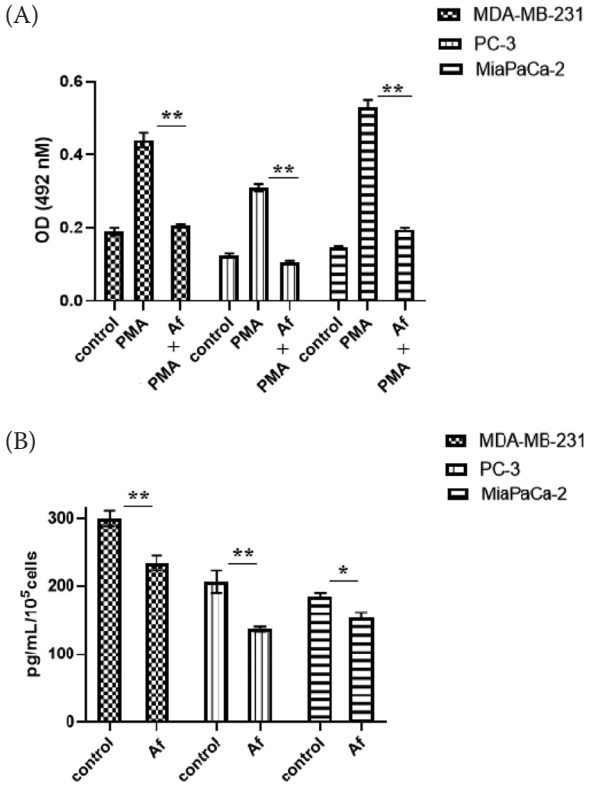


Figure 6. Inhibition of (A) NF-κB activation and (B) VEGF secretion by A. fragrantissima extract (250 µg/mL) in selected cancer cell lines (P < 0.01/*P < 0.5).**

angiogenesis, which was markedly associated with significant inhibition of the expression of pro-angiogenic cytokines, including VEGF *in vitro* and *in vivo* (Lugano *et al.*, 2020). Thus, we further explored the effect of extract on the expression of VEGF in selected cancer cell lines, and as demonstrated in Figure 6B, the extract displayed significant inhibitory potential against VEGF in these cells. Studies using adenoviral constructs that overexpressed IkappaBalpha (endogenous inhibitor of NF-κB) have demonstrated that the CD40L-induced VEGF production in macrophages is significantly inhibited following the infection with the inhibitor, indicating that VEGF expression is NF-κB dependent (Kiriakidis *et al.*, 2003). The ability of the *A. fragrantissima* extract to inhibit NF-κB exhibits its potential to interfere with oncogenic signaling cascades in cancer cells.

Conclusion

Natural compounds, which are either part of human nutrition or traditional medicine, have been extensively examined for apoptosis-inducing action, and experimental data were found to be consistent with the inhibition of growth in cancer cells *in vitro* and tumor progression *in vivo* (Figure 7). The results presented here clearly show that the hydro-alcoholic extract of *A. fragrantissima* effectively inhibits cellular proliferation and progressively

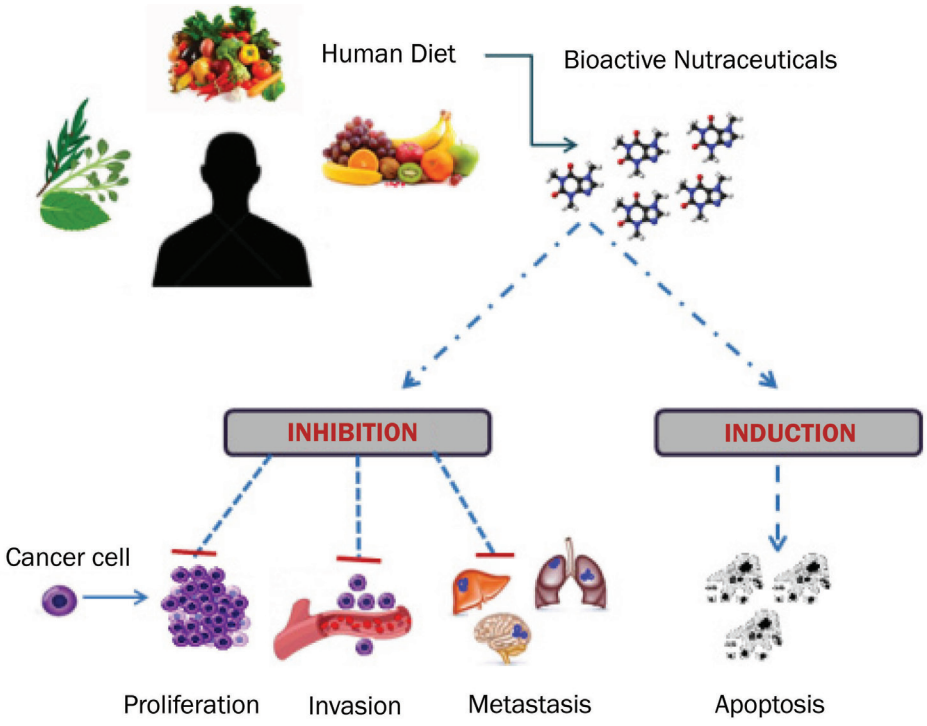


Figure 7. Schematic representation of anticancer actions of nutraceuticals in chemoprevention.

induces apoptotic cell death in human cancer cells. The mechanism of such an anti-cancer effect might involve inactivation of NF-κB signaling and the corresponding downregulation of its target cytokines such as VEGF. Cancer cells constitute an array of warped up signaling pathways, which are highly compromised, and food unlike drugs have an advantage of simultaneously influencing and interfering with these pathways due to multicomponent bioactivity. Moreover, it is understood that for such plant extracts, which are a mixture of various kinds of structurally diverse phytochemicals, the associated anticancer proprieties are difficult to be judiciously assigned to any particular compound. Thus, the extract needs further characterization for molecules responsible for the activity, which might lead to more potent pharmacological activity with isolated compounds alone or in synergism. However, the use of the *Achillea* species as functional food in the human population provides partial evidence of its health benefits and safety index. The dietary habits and the related health benefits vary across the continents and cultures. However, the precise identification of cause and effect relationship between diet and disease prevention as well as diet and gene function provides an opportunity for the development of functional foods for a uniform or personalized consumption by the population for their well-being or when stressed with undesired pathological and chronic state of health. Currently, when people are becoming more conscious of lifestyle and dietary behavior, the functional foods are expected to be of greater relevance wherein they are expected to have a significant place in disease prevention. The study holds significance in highlighting as a proof-of-concept, the importance of therapeutically active plant-derived molecules, which show some evidence-based outcome as functional food or ingredients of traditional medicine. Achillea species are used in various cultures as functional food for health benefits. The inferences drawn from the experimental profiling of the functional foods or nutraceuticals provide important milestones in encouraging the human population for preferentially adopting dietary habits, with evidence-based role in disease prevention. Nevertheless, food as a part of human culture has since ancient times provided sophisticated knowledge of associated health benefits and well-being among human population with varying dietary patterns based on ethnicity, sociocultural aspects, geographical location, and environmental cues.

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Conflict of interest

There is no conflict of interest to declare

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