ISOLATION OF GINGEROLS AND SHOGAOLS FROM GINGER AND EVALUATION OF THEIR CHEMOPREVENTIVE ACTIVITY ON PROSTATE CANCER CELLS AND ANTI-INFLAMMATORY EFFECT ON 12-*O*-TETRADECANOYL-PHORBOL-13-ACETATE (TPA)-INDUCED MOUSE EAR INFLAMMATION

by

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and approved by

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ASTRACT OF THE DISSERTATION

Isolation of Gingerols and Shogaols from Ginger and Evaluation of Their Chemopreventive Activity on Prostate Cancer Cells and Anti-inflammatory Effect on 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-Induced Mouse Ear Inflammation

by DIVYA RAMJI

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Ginger, obtained from the rhizome of *Zingiber officinale* (Family *Zingiberaceae*), has been used extensively as a spice and in traditional medicine. The compounds in ginger primarily responsible for its medicinal properties are the gingerols. Gingerols can undergo dehydration, during storage and processing, to form the corresponding shogaols. Studies conducted so far have primarily focused on the biological activities of ginger and 6-gingerol. The main objectives of this research were to evaluate the anti-inflammatory and chemopreventive activities of gingerols and shogaols.

The crude ginger extract was subjected to column chromatography to obtain a mixture of gingerols, a mixture of shogaols, 6- gingerol, 8-gingerol and 6-shogaol which

were characterized using high pressure liquid chromatography and nuclear magnetic resonance spectroscopy.

The anti-inflammatory activity of 6-gingerol and 6-shogaol was evaluated using the 12-*O*-tetradecanoylphorbol-13-acetate-induced mouse ear inflammatory model. Both 6-gingerol and 6-shogaol inhibited ear edema as well as the levels of proinflammatory cytokines.

The primary focus of this study was to evaluate the chemopreventive potential of these compounds on prostate cancer cells (LNCaP and PC-3). We hypothesized that gingerols and shogaols exhibit their chemopreventive potential through the modulation of the intrinsic pathway of apoptosis. Cell viability studies indicated that, among the compounds tested, 6-shogaol, 8-gingerol and shogaol mixture were the most effective in inhibiting cell growth in both cell lines. Morphological assessment, cell cycle, Annexin V staining and western blot analysis showed that 8-gingerol and 6-shogaol induced apoptosis in both the cell lines. Western blot analysis further confirmed our hypothesis that 8-gingerol and 6-shogaol induced apoptosis through activation of the intrinsic pathway of apoptosis as seen by caspase-9 cleavage. In addition, 8-gingerol and 6-shogaol induced the production of reactive oxygen species (ROS) which correlated well with the induction of apoptosis. This suggests that production of ROS could be one of the mechanisms of apoptosis induction by these compounds.

In conclusion, our study shows for the first time that gingerols and shogaols isolated from ginger were able to inhibit the growth of prostate cancer cells. 6-shogaol and 8-gingerol were able to induce apoptosis in both cell lines by activation of the intrinsic pathway of apoptosis.

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Introduction

Ginger, obtained from the rhizome of *Zingiber officinale* (Family *Zingiberaceae*) has been used as a spice and in traditional medicine due to its characteristic pleasant flavor, spicy taste and health beneficial properties [1-4]. The pleasant sweet citrus like aroma of the volatile oil of ginger has led to its wide popularity as a spice and flavorant in cooking [5,6]. In addition, fresh and dried ginger have been used as a stomachic, anti-emetic, cardiotonic, anti-diarrheal as well as in the treatment of arthritis and certain respiratory disorders in several traditional systems of medicine [7-11]. Most of the above pharmacological actions and the characteristic pungent taste of ginger are due to the composition of its oleoresin, primarily consisting of gingerols.

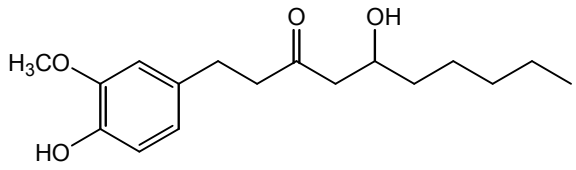
Chemistry of ginger

The chemistry of ginger has been widely investigated over the past several decades. The compounds in ginger primarily responsible for its characteristic spicy taste and pungency, such as gingerols, gingerdiols, zingerone and shogaols, make up almost 8% of the oleoresin [1,2,4,12,13].

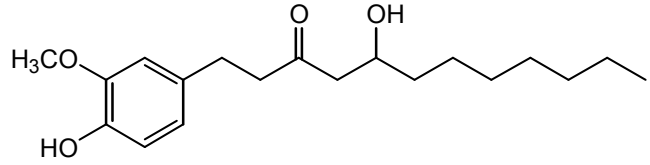
Gingerols are a homologous series of phenyl alkanones, primarily consisting of 6-, 8- and 10- gingerol (Figure 1), which differ in the length of their alkyl side chain. Among these, 6-gingerol is the most pungent (75% pungency) followed by 10-gingerol and 8-gingerol [5,14,15]. The content of gingerols, varies depending on the type of cultivar, variety and geographical location and is often used to estimate the freshness of ginger [16,17].

FIGURE 1

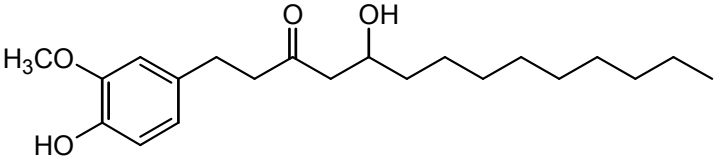
Chemical structures of the pungent components of ginger



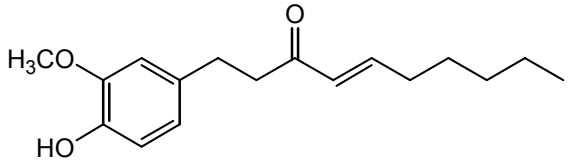
6-Gingerol



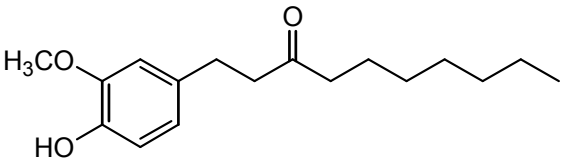
8-Gingerol



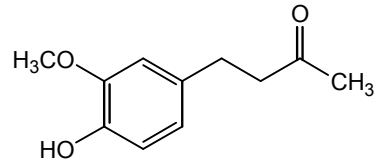
10-Gingerol



6-Shogaol



6-Paradol



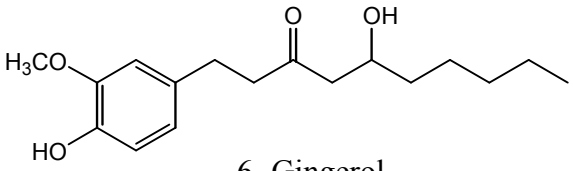
Zingerone

On storage or dehydration, gingerols can lose a molecule of water to form the corresponding shogaols as shown in Figure 2 [16]. The dehydration of 6-gingerol to 6-shogaol was shown to be favored at higher temperatures and acidic pH [3,15]. In addition, 6-gingerol can also undergo retro-aldol condensation to form zingerone. Gingerols, shogaols and zingerones are primarily responsible for the characteristic spicy pungent taste of ginger. In addition the oleoresin also contains, compounds such as gingenesulfonic acid, shagosulfonic acids and cyclic diaryl heptanoids which have been shown to contribute to the pharmacological activity of ginger [18-20].

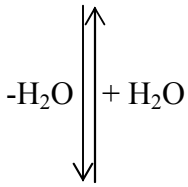
The volatile oil of ginger consists of a mixture of terpenes (including monoterpenes and sesquiterpenes) and nonterpenoid compounds which are responsible for the characteristic flavor and taste of ginger [5,21]. The main components responsible for the aroma of fresh ginger are geranial (citrus-like), geranial acetate (floral), geraniol (floral), neral, (citrus-like), linalool (floral), zingiberene (citrus-like), sesquiphellandrene and farnesene [5,22,23].

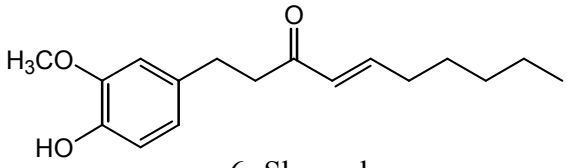
FIGURE 2

Degradation of gingerols during storage and processing

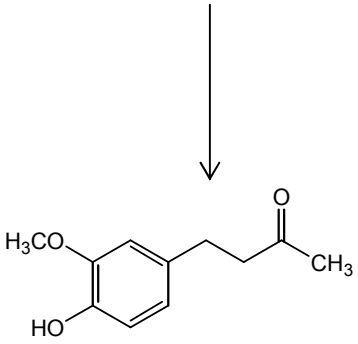


6- Gingerol





6- Shogaol



Zingerone

Biological effects of ginger

Ginger has been used for the treatment of several conditions such as cold, flu, gastrointestinal disturbances and arthritis in traditional systems of medicine. Recent studies have shown ginger to possess anti-oxidant, anti-inflammatory, chemopreventive, hypoglycemic, analgesic, antipyretic, anxiolytic, anti-emetic, antifungal and cardiovascular properties in both *in vivo* and *in vitro* models [9,11,24-41].

Gastrointestinal effects

Ginger exhibits several beneficial effects on the gastrointestinal tract and has been used for this purpose in several traditional systems of medicine. Ginger and its components (6-gingerol and 6-shogaol) have been shown to increase intestinal transport in mice by inhibiting muscle movement [42,43] and aid in digestion by enhancing vagal gastric nerve activity leading to activated gastric function [44]. Ginger has also been shown to increase the levels of pancreatic lipase, amylase, trypsin and chymotrypsin and the levels of intestinal lipase, amylase, alkaline phosphatase and acid phosphatase in rats thus aiding in the digestion of fats, proteins, starch and fiber [45]. The anti-diarrheal properties of ginger have been attributed to its ability to inhibit motility and smooth muscle contractions in isolated rat ileum [25,46]. In addition, ginger has been shown to possess anti-ulcer and hepatoprotective activity owing to its membrane stabilizing and anti-oxidant properties [11,28,47].

Ginger is also used commonly to treat nausea and vomiting caused due to motion sickness, pregnancy and other conditions. Its efficacy is attributed to the pungent principles and the volatile oils present in ginger, which act by stimulating tone and

peristalsis of the stomach [48]. Due to its direct effect on the gastrointestinal tract, ginger does not cause the common side effects, such as drowsiness, seen with commercially available anti-emetics. Several clinical studies have shown ginger to be beneficial in nausea and vomiting caused due to pregnancy, chemotherapy, motion sickness and other conditions [48-55].

Antioxidant effects

Free radicals are highly reactive chemical species, generated during respiration, oxidative phosphorylation reactions and inflammation, that have the capacity to cause cell and DNA damage [56]. The antioxidant defense mechanisms in the body, consisting of enzymes such as superoxide dismutase, glutathione peroxidase and catalase serve to inactivate these free radicals. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide and reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide when present in excessive amounts can result in oxidative stress leading to the development and promotion of several ailments such as cancer, inflammation and heart disease [57]. Antioxidants act by trapping these ROS and RNS, thereby reducing the oxidative stress. Dietary antioxidants such as vitamin C, tocopherols and certain phytochemicals such as tea have received tremendous attention due to this property and are therefore beneficial to health. The antioxidant properties of ginger have been investigated in several *in vitro* and *in vivo* studies [24,27,34,57-62]. Ginger has been shown to inhibit lipid peroxidation, superoxide dismutase activity, increase glutathione content as well as scavenge free radicals in animal models [24,34,57,63].

Anti-inflammatory activity

Ginger and its pungent components have been shown to possess anti-inflammatory properties in several *in vivo* and *in vitro* studies [31]. They have been shown to inhibit platelet aggregation and arachidonic acid metabolism (including cyclooxygenase (Cox) and lipoxygenase (Lox)) thus exhibiting anti-inflammatory, anti-pyretic and analgesic properties [26,33,64-70]. Ginger also exhibited a dose dependent inhibition of tumor necrosis factor-α (TNFα), nuclear factor-κB (NF-κB) DNA binding activity as well as degradation of inhibitory κB (IκB) *in-vitro* [37,71]. Ginger and its active component 6-gingerol have been shown to decrease Cox-2 expression and lower the production of eicosanoids in a dose dependent manner in mouse skin due to the down regulation of NF-κB and mitogen activated protein kinases (MAPK) [37,71]. In activated macrophages, 6-gingerol was shown to inhibit nitric oxide synthesis, lower the levels of inducible nitric oxide synthase protein and inhibit DNA damage induced by peroxy nitrite in a dose dependent manner [31,36].

Arthritis – Arthritis or inflammation of the joints affects nearly 1 in 3 adults in the United States of America and can occur in various forms such as osteoarthritis and rheumatoid arthritis [72,73]. The most common method of treating arthritis is with the use of anti-inflammatory agents. However they are slow acting and can cause long-term toxicity. Alternative medicine and herbal supplements are gaining popularity due to their safety and low toxicity. A few clinical trials have shown ginger to significantly reduce symptoms of knee osteoarthritis compared to placebo [74]. In addition *in vivo* and *in vitro* studies have also shown the anti arthritic effect of ginger. Ginger has also been shown to

decrease the levels of several pro-inflammatory mediators, which have been shown to be important in arthritis, such as prostaglandin E_2 (PGE_2) and TNF in an *in vitro* study using human synoviocyte cells. In addition ginger also suppressed the expression of Cox-2 and NF-κB in these cells [37]. 6-shogaol (a pungent constituent of ginger) was recently shown to decrease the chronic inflammatory response in arthritic knee of rats [75].

A few studies have shown ginger to possess pro-inflammatory properties. Ginger has been shown to induce the expression of nitric oxide synthase and increase the levels of nitric oxide in a mouse macrophage cell line [76]. However, 6-gingerol inhibited the formation of nitric oxide in LPS treated macrophages in a dose dependent manner [36].

Chemopreventive activity

Cancer is the second leading cause of death in the United states after heart disease [77]. The treatment of cancer usually involves radiation and chemotherapy. However in recent years, phytochemicals are being evaluated for their chemopreventive activity due to their strong antioxidant properties and fewer side effects. The chemopreventive activity of ginger has been established in a few *in vivo* and *in vitro* studies [30,78-84].

The chemopreventive activity of ginger has been evaluated in a few animal models of cancer. Ginger extract has been found to exert a protective effect on urinary bladder cancer in rats [85], 1,2-dimethylhydrazine induced colon cancer during the initiation and post-initiation stages in rats [86], spontaneous mammary gland tumorigenesis [87] and on skin carcinogenesis in mice [88-90]. Ginger extract was shown to dose dependently decrease the incidence of skin tumor caused by the dual application of DMBA and TPA. In addition, the authors have shown that ginger extract was able to

decrease the levels of several biochemical markers involved in carcinogenesis such as ornithine decarboxylase, cyclooxygenase and lipoxygenase in a dose dependent manner [88]. In a similar study 6-gingerol and 6-paradol were able to decrease the incidence of skin tumors and induction of ornithine decarboxylase activity in mice [89,90].

The chemopreventive activity of ginger has also been evaluated in a few *in vitro* studies. These studies have shown that ginger and some of its active components such as 6-gingerol, 6-shogaol and 6-paradol induce apoptosis and decrease cell growth in different cancer cell lines [91-98]. A recent study conducted on a hepatoma cell line concluded that 6-shogaol induced apoptosis by induction of reactive oxygen species leading to oxidative stress and activation of the caspase cascade [92]. A study conducted in Korea in 2005 showed that 6-gingerol was able to inhibit tumor growth by decreasing angiogenesis (formation of new blood vessels) *in vivo* as well as *in vitro* [99].

In addition, studies have also shown ginger to be effective in inhibiting other factors which lead to the development of cancer. Ginger extract, gingerols and 6-shogaol have been shown to inhibit the growth of *Helicobacter pylori* (a bacterium that has been known to be involved in the development of gastric cancer) *in vitro* thus exerting a chemopreventive effect [100].

Cardiovascular effects

Ginger and its components have been shown to exhibit cardio-protective activity through several mechanisms such as lowering of blood pressure and inhibition of arachidonic acid metabolism [101,102]. Metabolites of arachidonic acid such as thromboxanes are important in blood clotting and may promote ischemic heart disease.

Ginger and its active constituents such as gingerols, shogaols and paradols have been shown to inhibit arachidonic acid induced human platelet aggregation as well as Cox and thromboxane synthase activity *in vitro* and thus may be beneficial in heart disease [33,67,70].

Ginger and its components have been shown to dose dependently decrease the synthesis of cholesterol *in vivo* and *in vitro* [29,103]. It has also been shown to lower the levels of triglycerides, total cholesterol, low density and very low density lipoproteins in plasma and also decrease the oxidation and subsequent uptake of lipoprotein by atherosclerotic mice and cholesterol fed rabbits [104,105]. By all of the above mentioned mechanisms ginger could be beneficial in heart disease and for general cardiovascular health.

Anti-diabetic activity

The anti-diabetic activity of ginger has been shown in a few studies. Ginger has been shown to decrease fasting blood sugar levels and increase insulin concentration in streptozocin-induced diabetic rats, while having no effect on normal rats [103]. However, in another study, administration of ginger extract lead to a dose dependent decrease in glucose levels in both normal and diabetic rats [106]. In addition, several compounds isolated from ginger have been shown to inhibit the enzyme aldose reductase (which has been shown to play an important role in diabetic complications) *in vitro*. In addition, these compounds also inhibited lens galactitol accumulation in rats and sorbitol accumulation in isolated human erythrocytes. Interestingly, 6-gingerol and 6-shogaol did not show such potent activity in any of these assays [107].

Toxicological effects of ginger

In spite of the above beneficial health effects, some studies have shown ginger to possess certain harmful properties. Ginger and some of its constituents have been shown to be mutagenic in nature and induce chromosome breakage, somatic segregation, C-mitosis as well as multiple anaphases in bulbs of *Allium cepa* [108]. In addition, ginger extract was shown to be genotoxic to murine bone marrow cells [109]. In a study conducted in pregnant rats, there was an increase in the number of embryonic deaths and fetal re-absorption on administration of ginger [110]. However no evidence of maternal toxicity or teratological changes in fetus was observed. The surviving fetuses were heavier, which the authors suggest was probably due to increased fetal growth when compared to the control rats [110,111].

Ginger extract has been shown to be safe when administered to mice up to a concentration of 300 mg/kg body weight. However, on administration of 500 and 700 mg/kg body weight, a mortality rate of 50% and 100% was observed respectively [63]. Administration of an ethanolic ginger extract (upto 100 mg/kg body weight) by oral gavage did not lead to any significant changes in blood glucose levels, blood coagulation, blood pressure or heart rate [112]. 6-gingerol and 6-shogaol appear to be safe when administered orally to mice with LD_{50} of 250 and 687 mg/kg body weight respectively.

Bioavailability of ginger

Most studies conducted on the bioavailability of ginger, have focused on 6-gingerol. On oral administration to rats, 6-gingerol undergoes oxidation of its phenolic side chain and conjugation to primarily glucoronides in the liver [113,114]. Metabolites

such as vanillic acid and ferulic acid were also found in the urine, probably due to metabolism in the intestine. In addition, 6-gingerol and 6-shogaol were shown to be enzymatically reduced to gingerdiol and 6-paradol respectively, in isolated rat liver [115]. On intravenous administration, 6-gingerol was found to be rapidly cleared from the plasma with a half life of 7.23 minutes and less than 0.1% of the dose remained in the plasma after 30 minutes [116]. In rat liver microsomes, 6-shogoal was shown to be metabolized to 6-paradol through enzymatic reduction [115].

Cancer

Cancer is a hyper-proliferative disorder of the cells, characterized by uncontrolled cell growth and impaired cell division [117-119]. It is caused by a combination of several different events such as mutations, DNA lesions, activation of oncogenes and inactivation of tumor suppressor genes [119-121]. These events are thought to be caused due to exposures to several factors for extended periods of time. Some of these factors include personal habits (smoking, alcoholism, and a lethargic life style), environmental agents (occupational exposures, environmental pollution, radiation such as ultraviolet and ionizing radiation), genetic constitution, endogenous damage (such as oxidative stress, inflammation and infection), hormonal dysfunctions, chronic infections and food contaminants such as mycotoxins [117-133].

Diet and nutrition have been shown to play a major role in the development and progression of cancer and several other diseases. A diet rich in antioxidants, vitamins and fiber has been recommended for a healthy lifestyle [120,121,134,135]. It has been suggested that almost 30% of cancers occurring worldwide is due to the dietary pattern. Excessive intake of salt, in addition to causing hypertension has also been shown to increase the incidence of stomach cancers. Certain food additives such as saccharin have been shown in animal models to increase the risk of certain types of cancers. In addition a diet rich in animal fat and calories has been shown to hasten the process of carcinogenesis [120,129].

There are several mechanisms involved in the development and progression of cancer such as mutations, DNA lesions, impaired cell division, and impaired defense mechanisms. In normal cells there is a fine balance between growth-promoting and

growth-inhibiting signals which keep cell proliferation and cell differentiation under control [136]. However during cancer, due to mutations in genes controlling such processes, this fine balance may be lost, leading to uncontrolled cell division, loss of differentiation and disruption of normal programmed cell death processes [118,137].

The normal growth and differentiation of cells is controlled by a tightly regulated cell cycle, where DNA duplication and cell division occurs [137-139]. The cell cycle consists of two major steps, the S phase in which DNA synthesis takes place and the M phase where mitosis (or cell division) takes place. These 2 phases are separated by a gap phase (G_2) where the cells are preparing for cell division after undergoing DNA synthesis. The first gap phase occurs before the S phase (G_1), during which the cells get prepared to synthesize the DNA [138,139]. The progression of the cell from one stage to another is controlled by the formation, activation and subsequent degradation of a series of cyclins and cyclin dependent kinases (CDKs). Each stage of the cell cycle is controlled and kept in check by several genes so that damaged DNA does not progress through the cell cycle. However, in cancer cells due to mutations in several of the key genes involved, cells with damaged DNA may be able to proceed through the cell cycle, leading to genomic instability and cancer [120,137,140-149].

Some of the key genes involved in the progression of cancer are the proto-oncogenes, the tumor suppressor genes and DNA repair genes. The proto-oncogenes such as ras, myc and Bcl-2 play important roles in the control of cell proliferation, cell differentiation, cell cycle and apoptosis. Mutations in these genes lead to a gain in function. The tumor suppressor genes, such as *p*53 and *p*16, on the other hand, arrest cell progression through the cell cycle and block differentiation. One of the most widely

studied mutation involves the *p*53 gene that has been shown to occur in over 50% of all cancers [120,137,143,149]. During the cell cycle, DNA lesions (such as chromosome breaks) can be passed on during cell division leading to mutations and uncontrolled cell division thus aiding in the process of carcinogenesis [120].

The high mortality and low prognosis rate of cancer is most commonly due to the late detection [118]. The treatment of cancer primarily involves radiation and chemotherapy. However due to the diverse mechanisms involved in the pathogenesis and progression of cancer, it has been challenging to control this disease. Although the treatment of cancer has progressed over the past several years, it is not satisfactory as yet. Hence the term chemoprevention has been coined, which may be defined as administration of pharmaceuticals, dietary agents, phytochemicals, micronutrients or macronutrients to prevent, inhibit or delay cancer induction and progression [133,150]. Chemopreventive agents can act through several mechanisms, including (i) inhibition of mutation and cancer initiation in cells (inhibition of mutagen uptake and metabolism), (ii) inhibition of tumor promotion and progression (inhibition of cellular uptake and metabolism of carcinogens, control of gene expression, inhibition of certain key enzymes and receptors involved in cancer progression) and (iii) inhibition of invasion and metastasis (such as cell proliferation, apoptosis, angiogenesis and cell differentiation) [133]. One of the key mechanisms, being evaluated presently to screen the chemopreventive potential of new agents is their ability to induce apoptosis, which is discussed in detail in the following section.

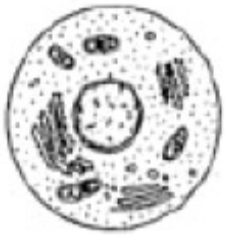
Apoptosis

Apoptosis, often referred to as programmed cell death, is an important and tightly regulated physiological process that regulates tissue homeostasis in normal cells [142,151-153]. This differs distinctly from another type of cell death known as necroses, where there is uncontrolled cell death leading to cell lysis and other serious health complications [154,155]. Several genes have been known to control the process of apoptosis, either through inhibition (such as Bcl-2) or induction (such as c-myc, *p*53, CDKs and certain members of the Bcl-2 family) [136]. The controlled process of apoptosis may be impaired in certain conditions which can lead to the development of cancer and neurodegenerative disorders [142,149,156].

There are several distinct biochemical changes that take place in a cell undergoing apoptosis, which manifests itself as morphological changes. Some of these include cell shrinkage, membrane blebbing, condensation and fragmentation of chromatin, shrinking and fragmentation of the nucleus leading to the formation of apoptotic bodies (as shown in Figure 3). These apoptotic bodies are eventually engulfed by surrounding cells, such as macrophages [142,151-162]. For this purpose, the apoptotic cells undergo translocation of the phosphatidyl proteins on their surface for easy recognition by the macrophages. During this process amino phospholipids such as phosphotidyl serines which are normally present in the inner leaflet of the cell membrane are translocated (with the help of scramblase) to the outer leaflet of the cell membrane [154,163-165]. This phenomenon is used for the early detection of apoptosis using Annexin V, a protein that specifically binds to phosphatidyl serine on the cell membrane and can be used as an early indicator of apoptosis.

FIGURE 3

Morphological changes occurring during apoptosis



Viable cell

Apoptosis
Cell shrinkage and chromatin condensation

Formation of apoptotic bodies

(Adapted from Geweis, 2003 [155] and Van Cruchten and W.Van den Broeck, 2002 [166])

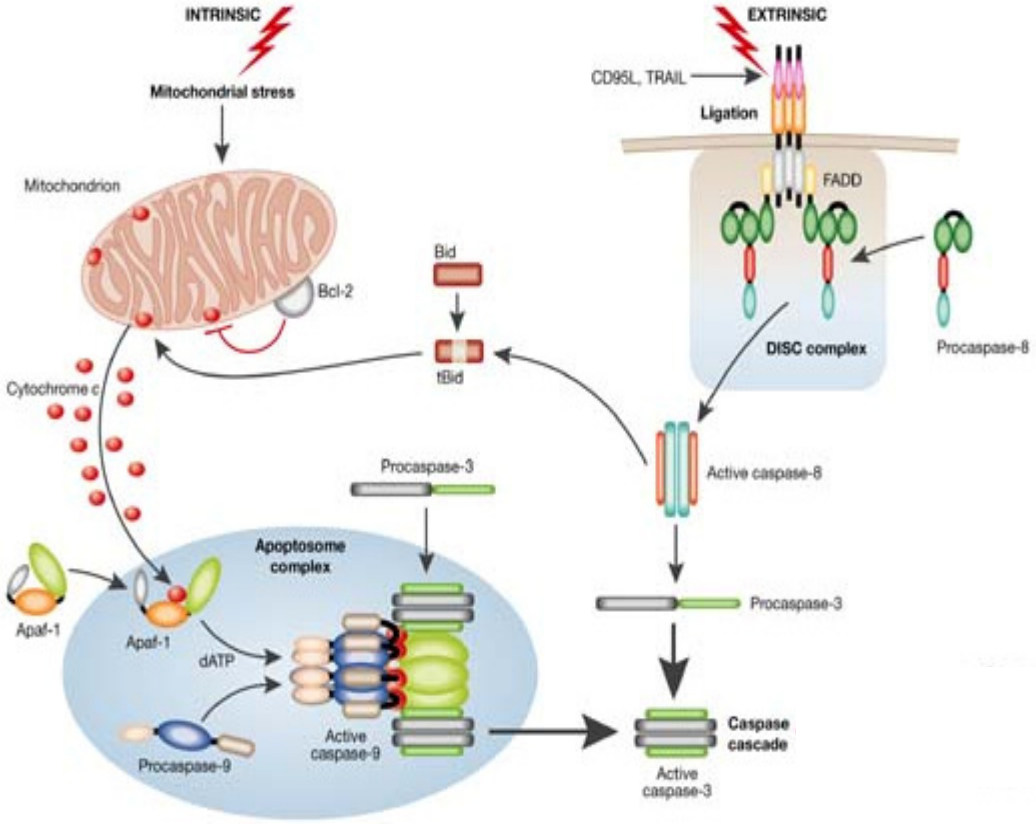
Apoptosis is primarily controlled by a family of proteases known as caspases (cystein aspartate- specific proteases) [158]. These are present in their inactive forms in the normal cell and require proteolytic processing for activation [153,158]. There are primarily two pathways of apoptotic cell death, the intrinsic or mitochondrial pathways and the extrinsic or death receptor pathway [142,153,167]. The activation of either of these pathways results in the cleavage of initiator caspases (such as caspase-9 for intrinsic pathway and caspase-8 for extrinsic pathway) leading to activation of effector caspases (such as caspases 3, 7, and 9) and finally apoptosis, as shown in Figure 4.

The extrinsic pathway is triggered by the activation and conformational changes on certain cell surface receptors (death receptors such as TNF and Fas/APO-1/CD-95 receptors), by the action of signaling molecules such as polypeptides, which have similar death domains and ligand binding activity [142,153,167] . Once a ligand binds to the receptor, it induces trimerization leading to the recruitment of adaptor proteins such as *Fas* activated death domain (FADD). This can interact with the initiator caspases (caspase-8 and -10) to form complexes commonly known as the death-inducing signaling complex (DISC). The initiator caspases can then lead to the proteolytic cleavage of effector caspases such as caspase-3. TRAIL (TNF-related apoptosis-inducing ligand) induces cell death only in transformed cells and not in normal cells [142,152-156,161,167-174].

The mitochondrial or intrinsic pathway is activated by intracellular signals, such as DNA damage and oxidative stress, leading to the release of cytochrome c from the mitochondria. This is mediated by the bcl-2 family of proteins [153]. Bax, a member of the Bcl2 family of proteins induces cytochrome c release resulting in loss of

FIGURE 4

PATHWAYS INVOLVED IN APOPTOSIS



MacFarlane and Williams, 2004 [175].

transmembrane potential and apoptosis. Bcl-2 and Bcl-X_L may inhibit apoptosis either by preventing cytochrome c release or its interaction with caspases. Cytochrome c in turn can bind with apoptotic protease activating factor-1 (Apaf-1) which results in the formation of apoptosome complex with caspase-9 leading to the activation of the caspase cascade. The activation of either caspase-8 or caspase-9 can lead to the proteolytic cleavage of downstream caspases including caspase-3, caspase-6 and caspase-7 [161,175]. This in turn can lead to the manifestation of other apoptotic specific features such as Parp (poly-ADP-ribose polymerase) cleavage and DNA fragmentation [151-161,169-172,175-177].

Reactive oxygen and nitrogen species (ROS/RNS) are also known to be involved in the process of apoptosis. During respiration, there is production of ROS such as hydrogen peroxide, hydroxyl radicals and superoxide anions under normal conditions. In cancer cells there is an increased production of ROS. During these situations, when there is an increased level of oxidative stress, the elevated levels of ROS can lead to cell death via apoptosis through the release of cytochrome c and activation of caspase-9 [154,161,178,179]. Elevated levels of ROS such as hydrogen peroxide and nitric oxide, has been shown to induce apoptosis. Also, elevated levels of glutathione, an antioxidant that scavenges free radicals in biological systems, has been shown to inhibit the process of apoptosis in isolated neutrophils and eosinophils [179].

Inflammation

Inflammation may be defined as the reaction or response of the body to tissue injury or damage [180]. Cell or tissue damage may be caused by several agents such as heat, chemicals, radiation, microbial infection, trauma as well as certain immunological processes. To counteract the tissue damage, body has two types of defense, the primary defense system (immediate such as inflammation) and the secondary defense system (controlled by the immunological system) [181]. The white blood cells or leukocytes play a very important role in the process of inflammation serving to attack and destroy the antigen. The reticuloendothelial cells such as phagocytes also form part of the primary defense system and are located at sites where attack is most likely to occur such as liver, spleen and bone marrow [181].

Inflammation is commonly characterized by four symptoms namely, redness, swelling, pain and heat [180,182]. During tissue injury, temporary vasoconstriction of blood vessels takes place followed by local vasodilatation and increased capillary permeability, which can increase blood flow through the area leading to redness and heat. During this process, due to an imbalance in capillary pressure, fluid can accumulate in the interstitial space leading to edema. Simultaneously, leukocytes migrate to the site of injury through a process of chemotaxis to attack the antigen. These processes are referred to as acute inflammation during which various mediators, ROS and RNS are released to help control the inflammatory process [180,183-186]. However if the acute inflammation persists over extended periods of time, due to dysregulation of various events, it can lead to the development of chronic inflammation. The basic mediators of inflammation and their functions are discussed in the following sections.

Cytokines

The cytokines are important intracellular messengers that play an important role in the inflammatory process [187]. They have a short duration of action and perform several diverse functions, such as growth regulation, cell division, inflammation and immunity, by interacting with specific receptors present in different cells [188,189]. Cytokines can be of different classes and possess pro- and anti-inflammatory properties. The interleukins (IL) are cytokines produced by leukocytes, which are important in the inflammatory process. The important pro-inflammatory cytokines include tumor necrosis factor α (TNF α), interleukin –1 (IL-1) and interleukin-6 (IL-6). TNF α is an essential cytokine, an endogenous tumor promoter and a key regulator of other cytokine production [190].

Interleukin-1, a pro-inflammatory cytokine produced by monocytes and macrophages, has been shown to induce other cytokine production, enhance phospholipase A_2 activation and production of prostaglandins [191]. There are two forms of IL-1: IL-1α and IL-1β, with similar physiological functions but different amino acid homology. They both exist in the precursor form and are activated during inflammation by proteases to participate in the inflammatory response [191]. Interleukin-6, a multifunctional pro-inflammatory cytokine produced by macrophages during inflammation stimulates the activation of T and B lymphocytes, induces fever and mediates acute systemic immune response [192,193]. In mouse it has been shown that PGE_2, IL-1 and TNFα can stimulate the production of IL-6 [191,192].

Eicosanoids

Eicosanoids are the lipid mediators of inflammation which are derived from fatty acids such as arachidonic acid (AA). Arachidonic acid is a polyunsaturated fatty acid (PUFA) that is mainly found esterified to the phospholipids in the cell membrane. Upon activation cellular phospholipases such as phospholipase A_2 (through the phosphorylation of mitogen activated protein kinase (MAPK)) act on phospholipids to release free arachidonic acid. This can then either be reincorporated into phospholipids or undergo metabolism through several pathways to generate a wide variety of lipid mediators such as prostaglandins (by Cox pathway) and leukotrienes (by Lox pathway), which serve as important intra- and intercellular messengers [194,195].

The Cox enzyme has been known to occur in three isoforms namely Cox-1, -2, -3. Cox-1 is an ubiquitous enzyme and expressed constitutively in the endothelium, kidneys, vascular smooth muscle and the gastrointestinal tract. The Cox-2 enzyme is inducible, as it is up-regulated during the process of inflammation by other inflammatory mediators [196]. Recently, another isozyme, Cox-3 has been identified in the central nervous system [197].

Lipoxygenases (Lox) are a group of iron containing dioxygenases which act by incorporating oxygen molecule into PUFAs at different positions to form various metabolites [198]. Its activity was first observed in 1975 in rabbit reticulocytes [199]. They exist in several isoforms (such as 5-, 12- and 15-Lox) giving rise to a myriad of products with different functions. 5-Lox is the most important of the lipoxygenases and converts AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosa-tetraenoic acid (5-HPTE), which further gives rise to leukotrienes B_4 (LTB_4).

Leukotrienes are known to play important roles in inflammatory diseases such as asthma [200]. LTB_4 is the most well studied metabolite of the Lox pathway and has been shown to be involved in the activation of inflammatory cells and assist in the migration of leukocytes to the site of inflammation.

The Cox and lox pathway of arachidonic acid metabolism, together produce a wide range of mediators which perform several critical functions in the body. These include their role in inflammation, blood clotting, maintenance of mucosal lining in stomach and as intra cellular messengers [198].

In certain cases, the inflammatory process is unable to function effectively and the cells of the inflammatory response are unable to remove the inciting stimuli leading to the development of chronic inflammation, that is characterized by sustained tissue damage leading to cellular hyper-proliferation, accumulation of inflammatory mediators, growth factors, ROS and RNS [179,201-204]. These in turn can cause DNA, protein and cell membrane damage ultimately leading to the development of diseases such as arthritis, ulcerative colitis, inflammatory bowel disease and cancer [159,198,202,205-208].

Inflammation and Cancer

The link between inflammation and cancer was first proposed in 1863 by Rudolph Virchow, who noted that cancer arises from sites of chronic inflammation [204-207,209-212]. In recent years, a strong link has been established between chronic inflammation and carcinogenesis in epidemiological, *in vivo* and *in vitro* studies. It has been noted that the inflammatory mediators, ROS and RNS are involved in the development of carcinogenesis by promoting cell growth, differentiation, metastasis and neoplastic progression [183,207].

In recent years it has been established that about 15% of cancers occurring worldwide are caused by chronic inflammation. Some of these include gastric cancer from *Helicobacter pylori*, hepatocellular carcinoma from hepatitis C, colorectal cancer from inflammatory bowel disease, lung cancer due to prolonged cigarette smoking, esophageal cancer from reflux esophagitis and mesothelioma from exposure to asbestos [205-210,213].

The arachidonic acid metabolites have received tremendous attention for their role in carcinogenesis. A link between the enzymes, metabolites and receptors involved in the Cox and Lox pathway has been established to inflammation and a variety of cancers. Aberrant arachidonic acid metabolism has been shown to play a major role in the development and progression of several cancers [195,204,214-243]. Both the Cox and the Lox pathway has been linked to cell proliferation, inhibition of apoptosis and promotion of angiogenesis [204]. In addition, several epidemiological, *in vitro* and *in vivo* studies have been conducted to demonstrate the chemopreventive activity of several Cox-2 and 5-Lox inhibitors on several models of cancer [196,198,204,227,239-254].

Peptide mediators of inflammation such as the cytokines and chemokines have also been shown to play an important role in the progression of cancer and have been shown to be expressed in several cancers such as breast, prostate, ovarian and bladder [210]. They can lead to tumor promotion either by stimulating cell growth and differentiation or by inhibiting apoptosis [202,255,256]. Cytokines such as tumor necrosis factor, IL-6 and IL-8 have been shown to promote tumor growth by activating transcription factors such as *ras* and NF-*k*B through tumor inflammation and vasculogenesis, causing DNA damage due to production of ROS/RNS, releasing growth factors to promote tumor growth, controlling tumor cell migration and leukocyte infiltration among many others [179,210,257-264].

The ROS/RNS that are released during inflammation (such as hydrogen peroxide, nitric oxide and superoxide anion) have also been shown to play a major role in the process of carcinogenesis. These free radicals have been shown to damage proteins, DNA, RNA and lipids leading to mutations in several key genes involved in carcinogenesis [159,179,208,265,266]. They have been shown to be involved in cell growth and tumor promotion by activating certain signal transduction pathways such as c-MYC, c-FOS, NF-κB and mitogen activated protein kinases (MAPK) [159,208].

On the other hand, certain free radicals such as hydrogen peroxide and superoxide anion are known to induce apoptosis through the activation of certain signal transduction pathways as mentioned earlier [179,183,184,267-269]. It has been hypothesized that ROS such as hydroxyl radical and nitric oxide can induce pores in the mitochondria leading to cytochrome c release and eventually apoptosis [179]. Nitric oxide has been shown to produce conflicting effects on apoptosis. In certain cells it has been shown to

inhibit apoptosis while in others it has been shown to induce the same (such as cancer, epithelial and endothelial cells). This inhibitory effect has been attributed to the capacity of nitrate to nitrosylate caspases and possibly inactivate them or to increase intracellular glutathione levels [179].

HYPOTHESIS

Phytochemicals are being evaluated for their chemopreventive activity in several *in vivo* and *in vitro* studies. Due to their safety and abundance in nature they provide a suitable alternative to commercially available drugs. The chemopreventive potential of phytochemicals has been extensively studied over the past several years [270-272].

Ginger has been used as a spice and in traditional medicine for several ages [8,273,274]. The chemopreventive activity of ginger has been evaluated over the past few years with promising results. Various *in vivo* studies have shown ginger to inhibit the size and number of tumors in several models. However, studies on the mechanism of action of ginger extract and its constituents are scarce. Only recently, studies have been conduced using primarily 6-gingerol in an attempt to evaluate the mechanisms of action. In this study we wanted to evaluate the anti-inflammatory and chemopreventive activities of gingerols and shogaols isolated from ginger.

The anti-inflammatory activity of gingerols and shogaols were evaluated using the 12-*O*-tetradecanoylphorbol-13-acetate-induced mouse ear inflammatory model. This model, developed in 1965, has been used extensively to screen the anti-inflammatory potential of several compounds [275-280]. During the early stages of development of this model, a 1% croton oil solution was applied to induce an inflammatory response in rats [275]. Presently, the active component of croton oil, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is being used for the same purpose. TPA has been shown to be a strong tumor promoter and induce hyperplasia, inflammation, over expression of cyclooxygenase and cytokines when applied to the mouse skin [264,279-282]. In the

present study we have used this model to evaluate the anti-inflammatory activity of gingerols and shogaols isolated from ginger. The effect of these compounds on TPA-induced ear edema and expression of cytokines was evaluated using the enzyme linked immunosorbant assay (ELISA).

The primary goal of this study was to study the chemopreventive activity of gingerols and shogaol on prostate cancer cells. Prostate cancer is the third most common cancer in men predominantly affecting older men in developed countries. In 2007, about 218,890 new cases and around 27,050 deaths are expected [283]. In the United States, it is the second leading cause of mortality in males [284,285]. Risk factors include high caloric intake, reduced physical activity and age. Micronutrients such as vitamins A, E and D, selenium, calcium, lycopene and phytoestrogens such as genistein, isoflavanoids and lignans have been shown to exert a protective effect in prostate cancer [286]. The treatment options available for this disease include androgen ablation therapy, prostatectomy and irradiation. These options are all localized to the prostate gland [287]. However, the mortality of prostate cancer results mainly due to metastasis (primarily to the bone and lymph nodes) and conversion from androgen dependent to independent form [287].

Under normal conditions prostate epithelial cells are dependent on androgens for their growth and survival and require binding of these androgens to the androgen receptor (AR). In the absence of androgens, these cells are unable to survive and undergo apoptosis [162,284,288,289]. However due to changes occurring during the process of carcinogenesis, prostate cancer cells can become androgen independent and thus gain resistance to chemopreventive drugs. Several mechanisms have been proposed through

which epithelial prostate cancer cells can survive in the absence of androgen. Some of these include increased sensitivity of the AR, increased production of androgens, mutations in the AR (multiple ligand binding sites on receptor, androgen substitution), activation of AR through other hormones and growth factors, activation of other pathways to completely bypass the AR pathway and alterations in the cell death regulatory mechanisms [162,284,288].

Several cell lines have been developed to study the mechanisms of prostate carcinogenesis such as LNCaP, PC-3 and DU-145. LNCaP cells (which are androgen-dependent in nature) were originally derived from the lymph node metastasis of a 55 year old man with prostate cancer in 1977 [290]. The PC-3 cells (androgen-independent) were derived from the bone metastasis of a 62 year old man with grade IV prostate cancer [291]. Both these cell lines have been used extensively to study the mechanisms of prostate carcinogenesis as well as to evaluate the chemopreventive activity of several compounds on prostate cancer [292-301]. In the present study, we wanted to determine the chemopreventive activity of gingerols and shogaols on prostate cancer cells, with particularly emphasis on apoptotic pathways. Hence we proposed the following hypothesis:

"Gingerols and shogaols exhibit their chemopreventive activity through induction of the intrinsic pathway of apoptosis."

In order to evaluate our hypothesis, we developed specific aims as outlined below:

1. To evaluate the effect of gingerols and shogaols on cell viability of LNCaP and PC-3 cells, using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].
2. To determine the effect of the test compounds on the induction of apoptosis.
3. To evaluate the effect of gingerols and shogaols on different phases of the cell cycle
4. To determine the molecular mechanisms of apoptosis induction using Western blot analysis.

MATERIALS AND METHODS

I. Isolation of gingerols and shogaols from ginger

Plant material, chemicals and reagents

Soft ginger extract in methanol was a gift from Sabinsa Corporation (Piscataway, NJ). Silica gel was purchased from Sorbent Technologies, Inc. (Atlanta, GA). Sephadex and Diaon HP-20 were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Isolation of gingerols and shogaols from ginger using column chromatography

Crude ginger extract was subjected to solvent extraction based on the method of Kiuchi *et al*. [69] prior to column chromatography. In short, the methanolic extract of ginger (50 g) was dissolved in ethyl acetate and partitioned with water. The ethyl acetate portion was collected, evaporated and the residue dissolved in methanol. This was further partitioned with hexane and the methanolic portion evaporated and used for the isolation of gingerols and shogaols.

The methanolic extract obtained by the above solvent extraction step was loaded onto a Sephadex column and eluted with 95% ethanol to yield gingerols, shogaols and related compounds. These extracts were analyzed by thin layer chromatography (using a 2:1 hexane: ethyl acetate solvent system) and similar fractions were combined. The extracts were then subjected to Diaon HP-20 column chromatography and eluted with 30%-70% ethanol. Similar fractions were combined to give rise to gingerol mixture and

shogaol mixture. The gingerol mixture was combined, evaporated to dryness under vacuum and the residue subjected to normal phase column chromatography using hexane: ethyl acetate as the solvent system to isolate 6-gingerol and 8-gingerol. For isolation of 6-shogaol, the shogaol mixture was subjected to reverse phase C18 column chromatography, and eluted with methanol-water solvent system.

The purity and identity of the isolated compounds were confirmed using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR).

Instrumentation

To analyze the purity of isolated compounds using HPLC a modified method of He *et al*. was employed [302]. A Varian Vista HPLC instrument with C-18 column (25cm x 46mm x 5 mm), obtained from Supelco (Belefonte, PA) attached to a variable UV detector was used for the analysis. A gradient elution program was followed as shown in Table 1. The purity of isolated compounds using NMR was conducted with the help of Dr. Shengmin Sang. ^{1}H (300 and 400 Hz) and ^{13}C NMR (100 Hz) were conducted using a Varian NMR spectrometer using deuterated chloroform ($CDCl_3$) as the solvent.

Table 1

HPLC program for the identification of gingerols and shogaols isolated from ginger

HPLC Instrument: Varian Vista 5500 LC

Column: Supelco Reversed Phase C-18 column (25cm X 46mm X 5 mm)

Injection volume: 20 µl

Flow rate: 1 mL/min

Detector: Spectroflow 783 variable wavelength detector at 280 nm

HPLC program:

Time (min)	Water (%)	Acetonitrile (%)
0	55	45
8	50	50
17	35	65
32	0	100
38	0	100
40	55	45

II. Evaluation of anti-inflammatory activities of gingerols and shogaols using the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear inflammatory model

Animals and Chemicals

Female CD-1 mice (3-4 weeks old; Charles River breeding laboratories, Kingston, NY) were housed six per cage in a room with controlled temperature and humidity with 12 hour light: dark cycles. All animals were given Purina Laboratory Chow 5001 diet (Ralston-Purina, St. Louis, MO) and water ad libitum. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) ELISA kits were purchased from R&D systems Inc. (Minneapolis, MN). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Effect of gingerols and shogaols on TPA-induced ear inflammation

Both ears of female CD-1 mice were treated topically with 10 μL acetone (negative control) or test compound in acetone 20 minutes prior to the application of acetone (negative control) or 1.5 nmol TPA (positive control and treatment groups) in acetone. The mice were sacrificed 6 hours after the last dose of TPA. Ear punches (6 mm in diameter) from each group were weighed, pooled together and homogenized in phosphate buffered saline (PBS) containing 0.4 M sodium chloride, 0.05% Tween-20, 0.5% bovine serum albumin (BSA), 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium, 10 mM ethylene diamine tetraacetic acid, and 20 U aprotinin per mL. The homogenates were centrifuged at 12000 x g for 60 min at 4°C and the supernatant was used for the determination of cytokine levels using a two-site sandwich ELISA.

Quantification of IL-1β and IL-6 using ELISA in mouse ears

The cytokine levels were measured using the ELISA kit according to manufacturer's protocol. In brief, the 96-well microplate was coated with 100 μl of capture antibody (IL-1β or IL-6) and incubated overnight at room temperature. The wells were washed with buffer (0.05% tween 20 in PBS) five times, blocked with 300 μL of blocking buffer (1% BSA, 5% sucrose and 0.05% sodium azide in PBS) and incubated at room temperature for 1 hour. The wells were washed and 100 μL samples or standards were added. The plate was incubated at room temperature for 2 hours, washed and treated with 100 μl of biotinylated goat anti-mouse secondary antibody before incubation at room temperature for 2 hours. The plate was washed, treated with streptavidin-horseradish peroxidase and incubated at room temperature in the dark for 20 minutes. The plate was washed again and treated with 100 μl substrate solution for color development for 20 minutes at room temperature. To this 2N sulfuric acid was added to stop the reaction and the optical density was measured at 450 nm using a microplate reader.

III. Evaluation of chemopreventive activities of gingerols and shogaols on prostate cancer cell lines (LNCaP and PC-3)

Chemicals

The test compounds (gingerol mixture, shogaol mixture, 6-gingerol, 8-gingerol and 6-shogaol) were isolated in the laboratory as described previously. RPMI 1640 (cell culture media), fetal bovine serum, trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO). Penicillin-streptomycin cocktail was purchased from GIBCO BRL (Grand Island, NY). Methanol, acetone and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). BCA protein assay kit was purchased from Pierce (Rockford IL). Sample buffer, polyacrylamide, nitrocellulose membrane and filter paper were purchased from Biorad Laboratories (Hercules, CA). Apoptosis detection kit for AnnexinV-FITC was purchased from R&D Systems (Minnepolis, MN). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Cell lines

Prostate cancer (LNCaP and PC-3) cell lines were obtained as a gift from Dr. Alan Conney's lab in the Laboratory for Cancer Research, Rutgers University. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin in a 5% carbon dioxide atmosphere at 37 °C and 95% relative humidity. The cells were sub cultured every 48 hours.

Assessment of cell viability

The effect of gingerols and shogaols on viability of prostate cancer cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [247,303,304]. LNCaP and PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C in a 5% carbon dioxide atmosphere for 24 hours to allow the cells to attach. The cells were then treated with different concentrations of the test compound and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with 0.5 mg/mL MTT solution and incubated for 4-5 hours. The resulting formazan product was solubilized in DMSO and the absorbance was measured at 570 nm.

Effect of gingerols and shogaols on prostate cancer cell morphology

LNCaP and PC-3 cells were seeded at a density of 5x10^4 cells/mL in 12 well tissue culture plates and incubated for 24 hours. The cells were then treated with different concentrations of the test compounds and incubated for an additional 48 hours. The cells were viewed under an inverted microscope at 10x to observe the morphology.

Assessment of apoptotic cell morphology

The effect of gingerols and shogaols on apoptosis in prostate cancer cells was evaluated after staining with propidium iodide (PI) using fluorescence microscopy as described previously [305]. Briefly, LNCaP and PC-3 cells were seeded at a density of 2 x 10^4 cells/mL in 6-well tissue culture plates and incubated at 37 °C for 24 hours. The cells were then treated with the test compounds and incubated for an additional 48 hours.

At the end of the treatment, the supernatant was removed from each well and the cells were fixed with a 1:1 mixture of acetone: methanol (1 mL/well) for 10 minutes. The cells were then incubated with 1 mL of propidium iodide (1 μg/mL) in PBS for 10 minutes at room temperature in the dark and viewed under a fluorescent microscope (Nikon Eclipse TE200). Photographs were taken using Image-pro plus software (Media Cybernetics, Silver Springs, MD). Apoptotic cells were characterized by condensed and fragmented nuclei.

Cell cycle analysis

The effect of gingerols and shogaols on different phases of the cell cycle was evaluated using flow cytometry as described previously [305-307]. LNCaP and PC-3 cells were plated at a density of 2 x 10^5 cells in 100 mm^2 Petri dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with the test compounds and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice, treated with 2 mL of ice cold ethanol and placed on ice for 30 minutes. The cells were then washed again with PBS to remove any traces of ethanol, treated with 0.1 μg/mL RNAse A and 10 μg/mL propidium iodide solution and incubated for 30 minutes in the dark. The cells were analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells in each phase of the cell cycle was calculated using cytologic software from Coulter Corp.

Annexin V-FITC and Propidium iodide (PI) co-staining for assessment of apoptosis

Effect of gingerols and shogaols on apoptosis in prostate cancer cells was evaluated using Annexin V reagent as described previously [308-310]. LNCaP and PC-3 cells were plated at a concentration of 2 x 10^5 cells/well in 6 well tissue culture plates and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with the respective compounds and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 μg/mL) and PI (5 μg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated using cytologic software from Coulter Corp.

Western Blot analysis

LNCaP and PC-3 cells were plated at a density of 5 x 10^5 cells/dish in 100 mm^2 tissue culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with the respective compounds and incubated for different time periods. At the end of the treatment the cells were harvested using RIPA lysis buffer (containing 1% protease and phosphatase inhibitor cocktails) and placed on ice for 30 minutes. The samples were then centrifuged at 12,000 x *g* for 15 minutes at 4°C and the protein concentration was determined using the Bio-Rad protein assay kit. The samples were stored at -80° C until further analysis.

Prior to performing polyacrylamide gel electrophoresis (PAGE), samples containing equal amounts of protein (20-40 μg) were boiled with sample buffer for 10

minutes at 95-100° C, centrifuged at 12,000 x *g* for 5 minutes and placed on ice. These samples were then electrophoresed using a freshly prepared 8-14% polyacrylamide gel under constant voltage (70-120 V). The proteins were transferred from the gel onto a nitrocellulose membrane for 2 hours at 100 Volts. The membrane was blocked using blocking buffer (Licor Biosciences, NE) overnight at 4 °C and incubated with primary antibodies for 12-18 hours at 4 °C. The membrane was then washed in PBS containing 0.1% tween-20 four times and incubated with infrared fluorescent-labeled secondary antibody (Licor Biosciences, NE) for 1 hour at room temperature. The membrane was washed again and the immunoblots were processed and visualized using the Odyssey infra red imaging system (Licor Biociences, NE) [311].

Determination of ROS

Measurement of intracellular ROS was performed as described previously [92,312-315]. PC-3 and LNCaP cells were plated on 100 mm^2 Petri dishes and incubated overnight at 37 °C and 5% CO_2 atmosphere. The cells (3 x 10^6 cells/mL) were treated with 5 μM 2',7'-dichlorofluorescein diacetate for 15 minutes in a shaking water bath. The cells were then treated with the respective test compounds and incubated for an additional hour. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp.

Statistical analysis

Statistical analysis was performed using Student's *t* test

RESULTS

I. Isolation of gingerols and shogaols from ginger

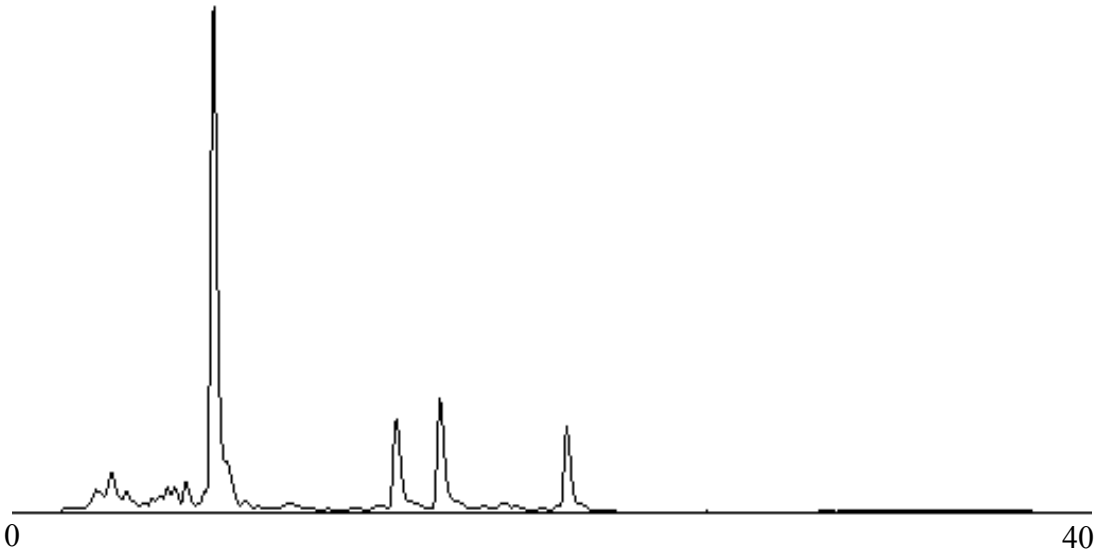
Ginger extract was subjected to solvent extraction and column chromatography as described previously to obtain the test compounds. On subjecting the crude ginger extract to Sephadex and Diaon HP-20 column chromatography, gingerol mixture and shogaol mixture was obtained. These mixtures were characterized using HPLC (according to the conditions described in Table 1) and the representative chromatograms are shown in Figures 5 and 12 respectively. The chromatogram of gingerol mixture showed the presence of four distinct peaks at retention times of 8.4, 15.9, 17.7 and 23 minutes. The first three peaks were identified as 6-gingerol, 8-gingerol and 10-gingerol respectively. The main constituent of gingerol mixture was 6-gingerol which made up almost 60% of its composition. The gingerol mixture was further subjected to normal phase column chromatography to obtain 6-gingerol and 8-gingerol. The purity of these compounds was verified using HPLC and NMR and the results are shown in Figures 6-11.

6-gingerol was obtained as a yellow oily liquid with a molecular weight of 294.39. The HPLC chromatogram (Figure 6) showed the presence of only one peak at 8 minutes which was identified as 6-gingerol using NMR spectroscopy.

The ^{1}H NMR of 6-gingerol ($CDCl_3$, 300 Hz, Figure 7). δ: 0.88 (3H, s, H-21), 1.257-1.280 (2H, d, J = 69 Hz, H-18 and H-19), 2.728-2.750 (2H, d, J = 6.6 Hz, H-14), 2.806- 2.830 (2H, d, J = 7.2 Hz, H-11), 3.854-3.864 (3H, d, J = 3 Hz, H-8), 5.534 (1H, s, 9-H and 16-H), 6.670 (1H, s, H-4), 6.806 (1H, s, H-6).

FIGURE 5

HPLC chromatogram of gingerol mixture

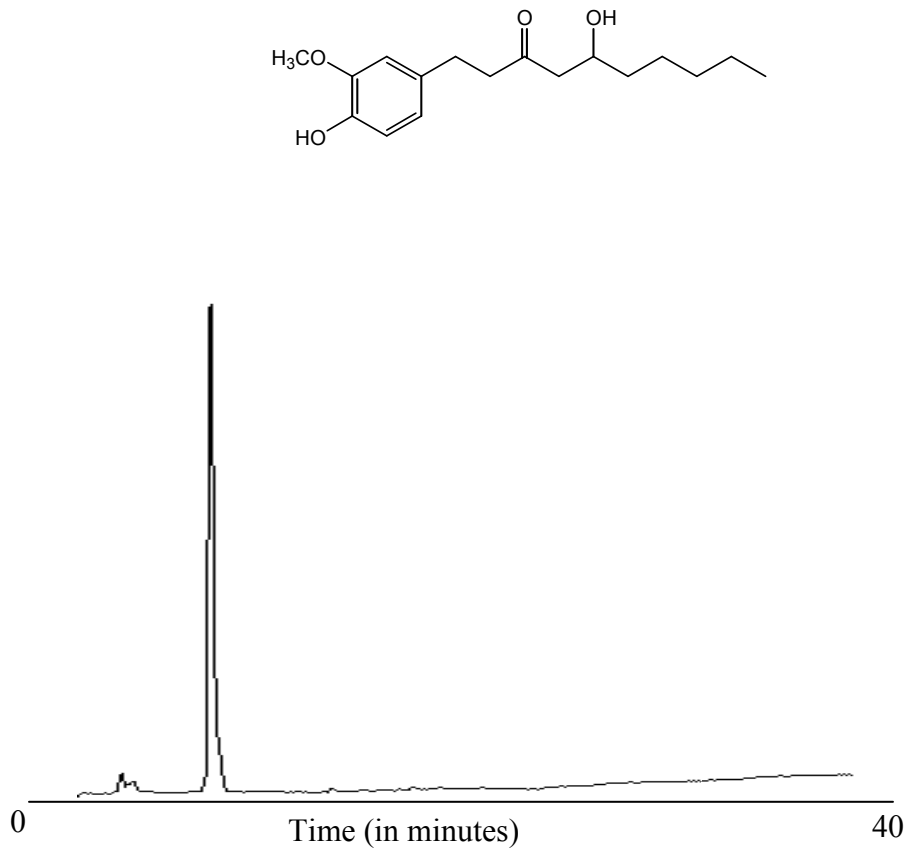


Peak No.	Retention Time	Compound	Composition (%)
1.	8.4	6-gingerol	58.72
2	15.9	8-gingerol	12.45
3	17.7	10-gingerol	14.32
4	23.0	Unknown	9.13

Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined to obtain gingerol mixture which was subjected to HPLC analysis, according to the method shown in Table 1.

FIGURE 6

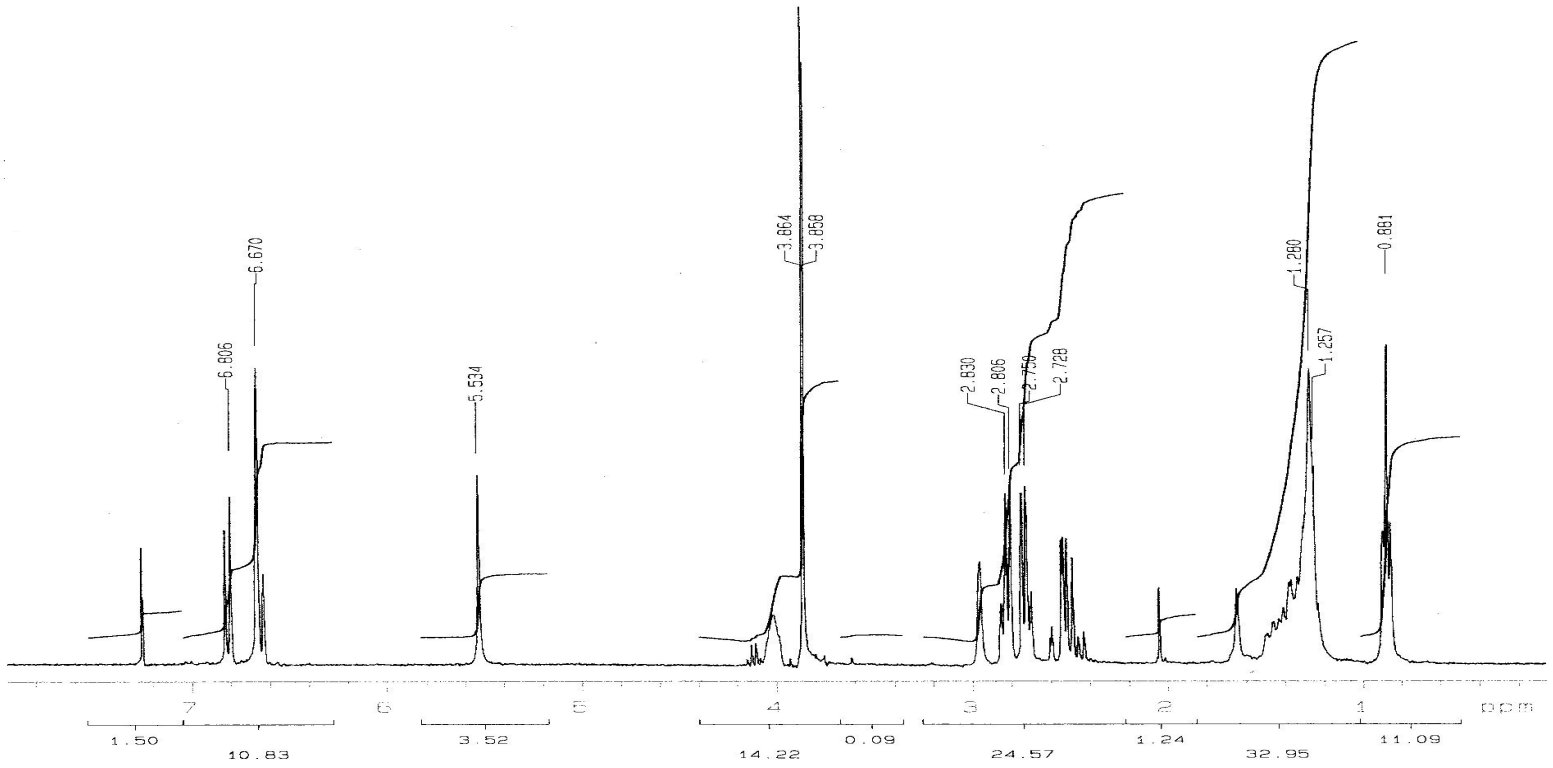
HPLC chromatogram of 6-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 6-gingerol. The purity of 6-gingerol was then confirmed using HPLC according to the method shown in Table 1.

FIGURE 7

^{1}H-NMR of 6-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 6-gingerol. The purity and identity of 6-gingerol was then verified using ^{1}H NMR ($CDCl_3$, 300 Hz).

^{13}C NMR of 6-gingerol (CDCl3, 100 Hz, Figure 8). δ: 211.46 (s, C-12), 146.68 (s, C-1), 144.25 (s, C-2), 132.86 (s, C-5), 120.98 (s, C-4), 114.62 (s, C-3), 111.28 (s, C-6), 77.21 (t, C-15), 67.92 (s, C-14), 56.13 (d, C-110, 49.63 (s, C-8), 45.66 (s, C-17), 31.95 (s, C-10), 29.55 (s, C-18), 25.34 (s, C-19), 22.78 (s, C-20), 14.19 (s, C-21).

8-gingerol was obtained as a yellow oily liquid with a molecular weight of 322.44. The HPLC chromatogram (Figure 9) showed a major peak at around 14 minutes which was identified as 8-gingerol using NMR spectroscopy.

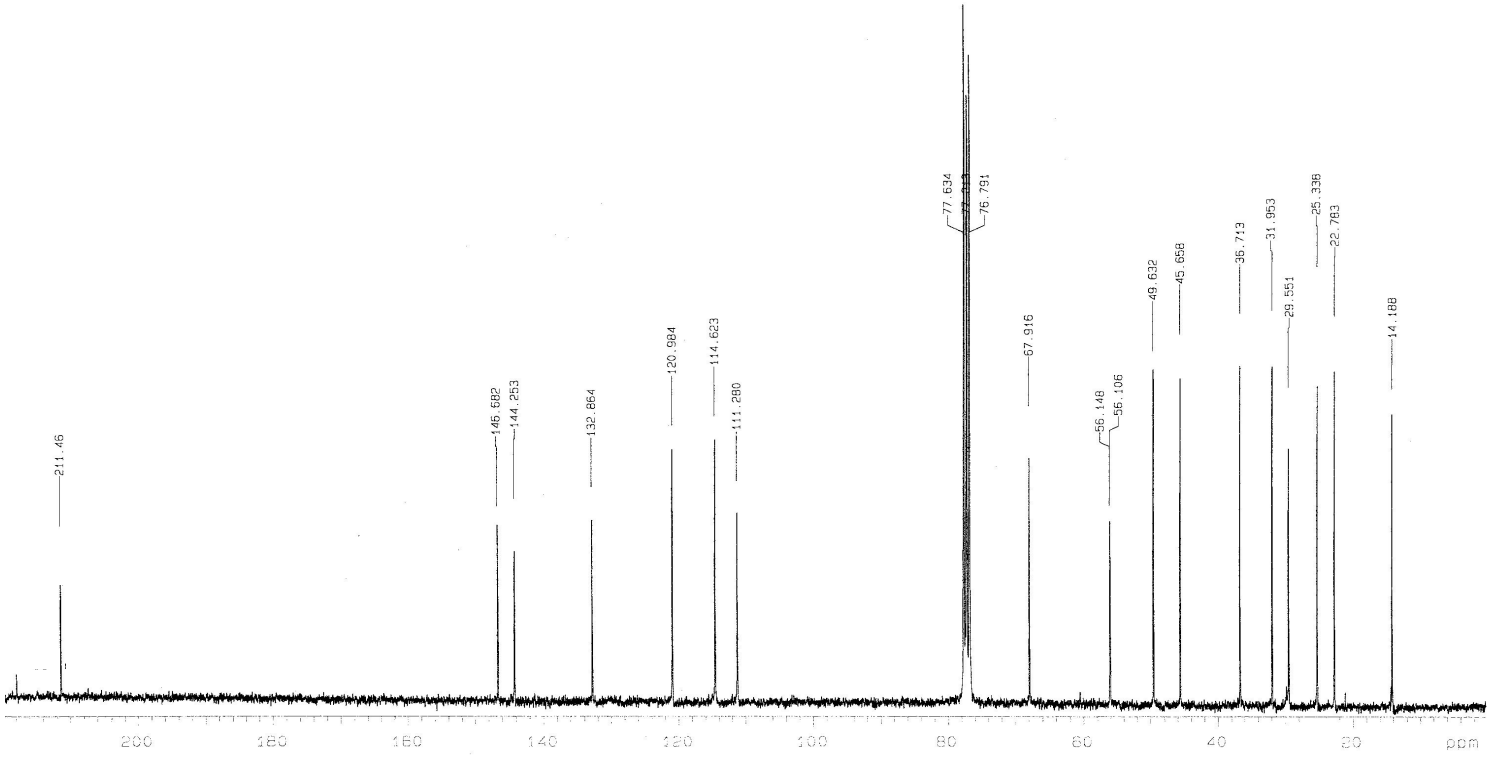
The ^{1}H NMR of 8-gingerol ($CDCl_3$, 400 Hz, Figure 10). δ: 0.88-0.91 (3H, t, *J* = 7.2 and 6.4 Hz, H-23), 1.29-1.57 (2H, m, H-18, H-19 and H-20), 2.47-2.62 (2H, m, H-14), 2.73-2.77 (2H, t, *J* = 7.6 and 6.8 Hz, H-11), 2.84-2.87 (2H, t, *J* = 6 and 7.2, H-10), 3.89 (3H, s, H-8), 4.04-4.07 (1H, d, *J* = 9.2, H-15), 5.58 (1H, s, H-9 and H-16), 6.67-6.70 (1H, t, *J* = 8 and 3.2 Hz, H-4), 6.83-6.85 (1H, d, *J* = 7.6 and H-6), 7.29 (1H, t, H-3).

^{13}C NMR of 8-gingerol (CDCl3, 100 Hz, Figure 11). δ: 211.31 (s, C-13), 146.18 (s, C-2), 143.69 (s, C-3), 132.39 (s, C-6), 120.46 (s, C-5), 114.13 (s, C-4), 110.71 (s, C-1), 76.80 (t, C-16), 67.41 (s, C-14), 55.62 (s, C-11), 49.08 (s, C-8), 45.18 (s, C-17), 36.19 (s, C-10), 31.56 (s, C-18), 29.13 (t, C-19 and C-20), 25.22 (s, C-21), 22.41 (s, C-22), 13.87 (s, C-23)

The HPLC chromatogram of shogaol mixture is shown in Figure 12. The chromatogram shows the presence of multiple peaks and the peak with a retention time of 16.33 minutes was identified as 6-shogaol. This was the major peak of shogaol mixture making up almost 45% of its composition. This was further subjected to reverse phase

FIGURE 8

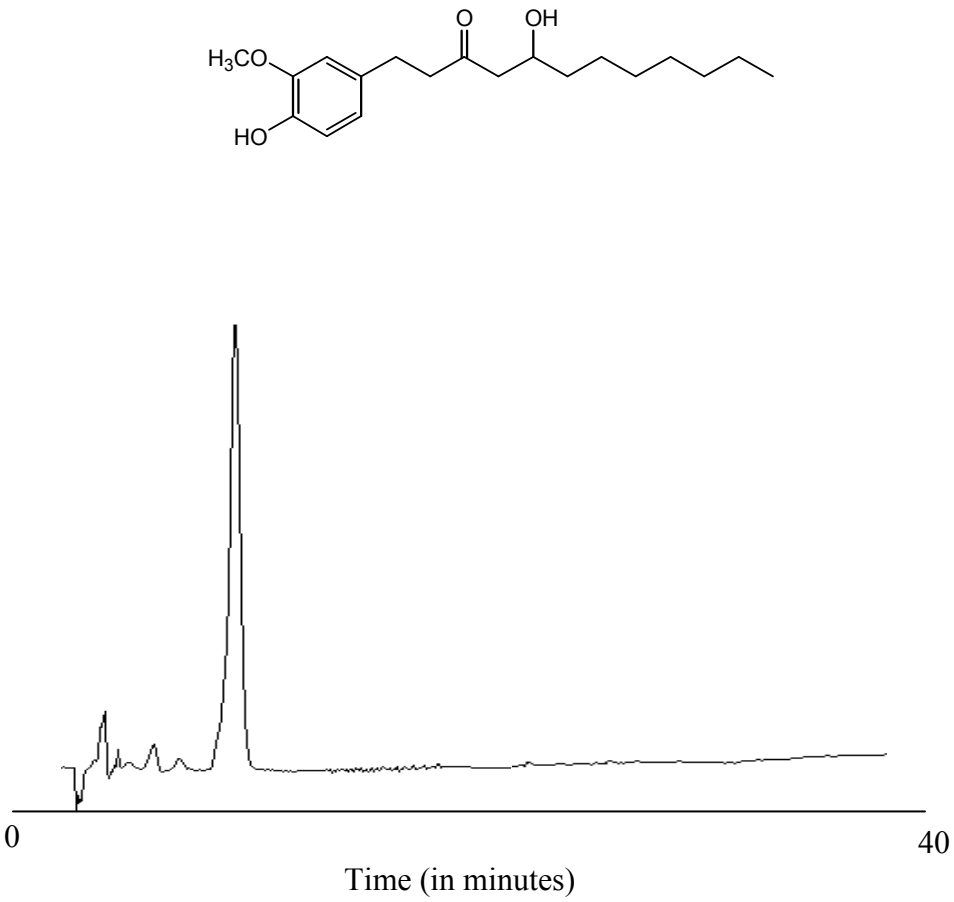
^{13}C-NMR of 6-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 6-gingerol. The purity and identity of 6-gingerol was then verified using ^{13}C NMR ($CDCl_3$, 100 Hz).

FIGURE 9

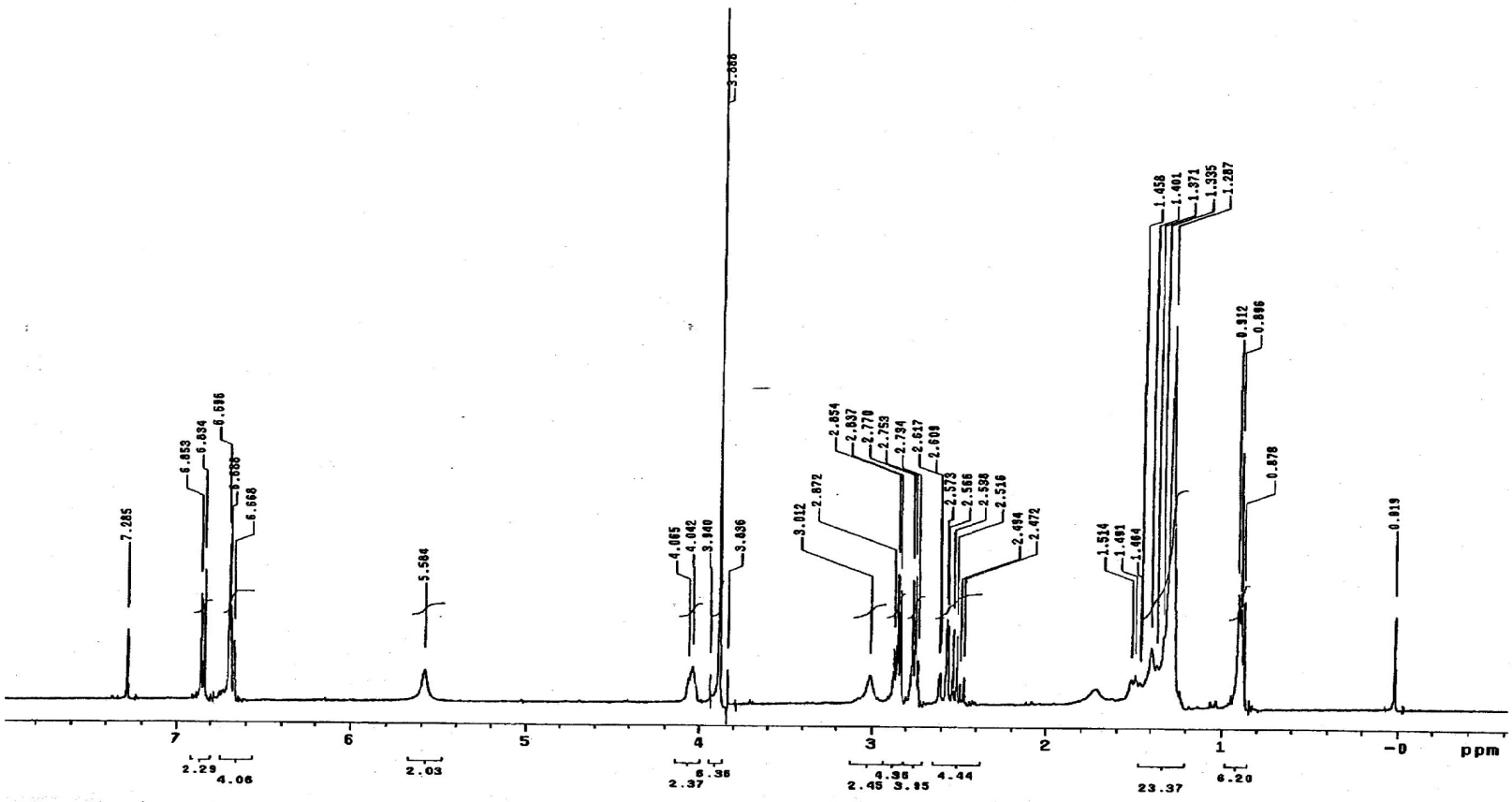
HPLC chromatogram of 8-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 8-gingerol. The purity of 8-gingerol was then confirmed using HPLC according to the method shown in Table 1.

FIGURE 10

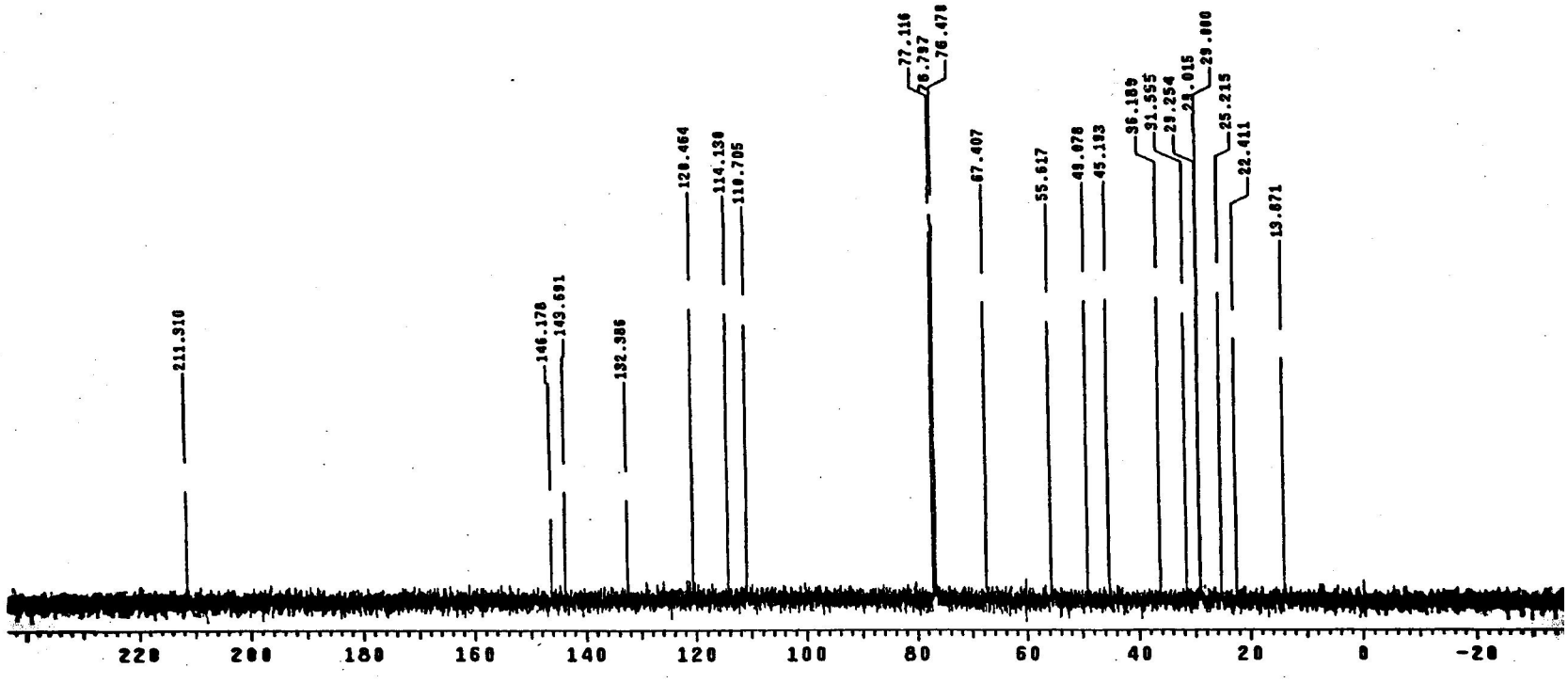
^{1}H-NMR of 8-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 8-gingerol. The purity and identity of 8-gingerol was then verified using ^{1}H NMR ($CDCl_3$, 400 Hz).

FIGURE 11

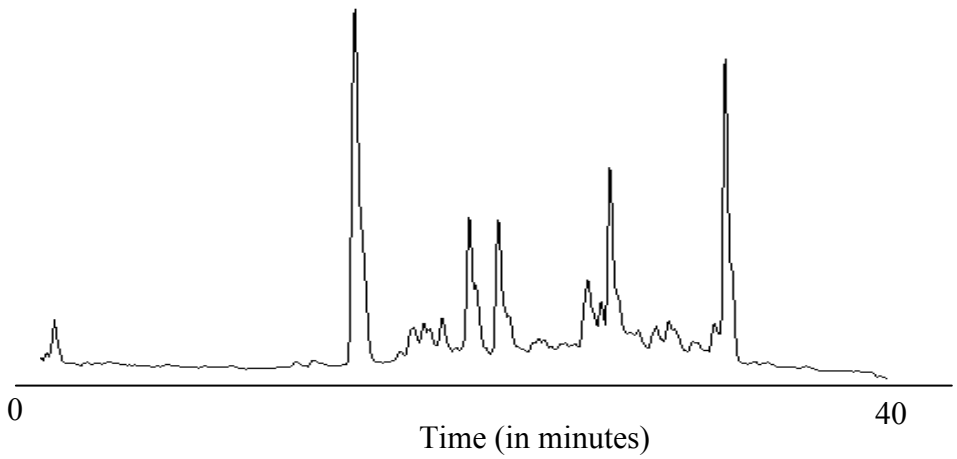
^{13}C-NMR of 8-gingerol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The gingerol mixture so obtained was subjected to normal phase column chromatography to obtain 8-gingerol. The purity and identity of 8-gingerol was then verified using ^{13}C NMR ($CDCl_3$, 100 Hz).

Figure 12

HPLC chromatogram of shogaol mixture



Peak No.	Retention Time (min)	Compound	Composition (%)
1	16.33	6-shogaol	44.92
2	22.30	Unknown	4.22
3	23.82	8-shogaol	8.47
4	28.47	Unknown	7.03
5	29.62	Unknown	13.50
6	35.62	10-shogaol	21.86

Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined to obtain shogaol mixture which subjected to HPLC analysis according to the method shown in Table1.

column chromatography to obtain 6-shogaol. The purity was verified using HPLC and NMR as shown in Figures 13-15.

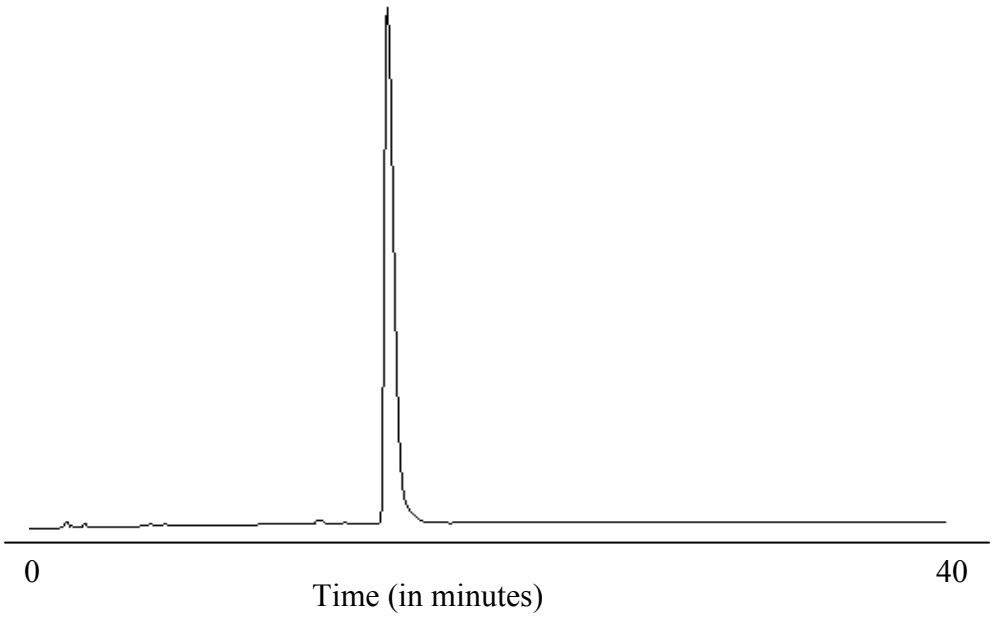
6-shogaol was obtained as a bright yellow oily liquid with a molecular weight of 276.37. The HPLC chromatogram (Figure 13) shows the presence of only one peak at a retention time of about 18 minutes which was identified as 6-shogaol using NMR spectroscopy.

The ^{1}H NMR of 6-shogaol ($CDCl_3$, 400 Hz, Figure 14). δ: 0.86-0.90 (3H, dd, *J* = 7.2 and 5.2, H-21), 1.26-1.31 (2H, m, H-18), 1.31-1.45 (2H, dd, *J* = 7.2 and 7.6 Hz, H-19), 2.17-2.18 (2H, d, *J* = 6.8 Hz, H-10 and H-11), 2.83-2.84 (H, t, *J* = 1.6 and 4 Hz, H-14 and H-15), 3.81-3.83 (3H, t, *J* = 2 and 2.8 Hz, H-8), 6.06-6.11 (1H, m, H-9), 6.65-6.70 (1H, dd, *J* = 8 and 2, H-4 and H-6), 6.80-6.84 (1H, m, H-3).

^{13}C NMR of 6-shogaol ($CDCl_3$, 100 Hz, Figure 15). δ: 199.83 (s, C-13), 146.32 (s, C-2), 143.72 (s, C-3), 132.84 (s, C-6), 120.48 (s, C-5), 114.23 (s, C-4), 111.00 (s, C-1), 147.83 (s, C-14), 130.0 (s, C-15), 55.55 (s, C-11), 41.63 (s, C-8), 32.19 (s, C-10), 31.07 (s, C-16), 29.61 (s, C-17), 27.48 (s, C-18), 22.16 (s, C-19), 13.70 (s, C-20).

FIGURE 13

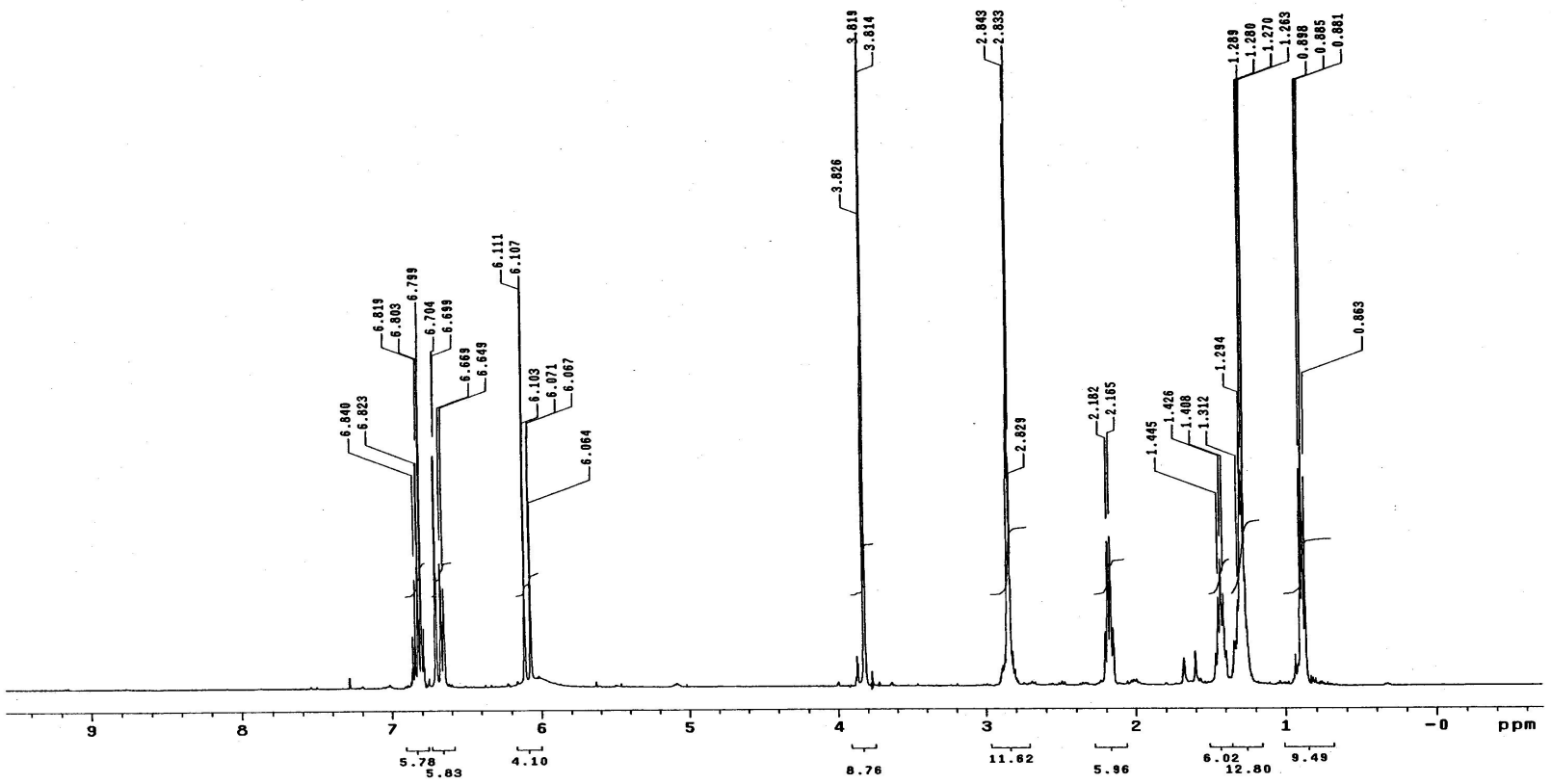
HPLC chromatogram of 6-shogaol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated, subjected to sephadex column chromatography. The resulting fractions were combined and subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The shogaol mixture so obtained was subjected to reverse phase column chromatography to obtain 6-shogaol. The purity of 6-shogaol was then confirmed using HPLC according to the method shown in Table 1.

FIGURE 14

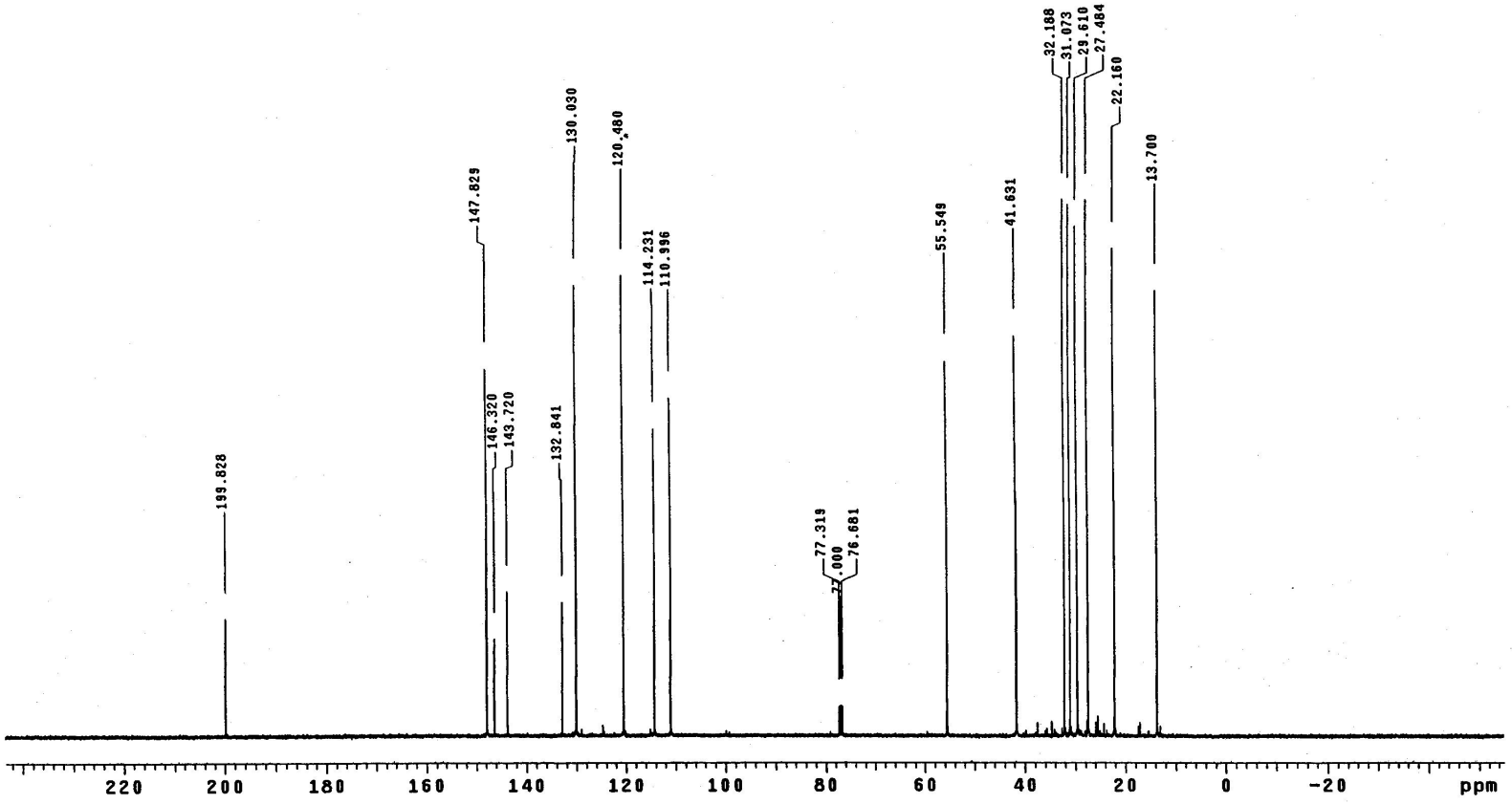
^{1}H NMR of 6-shogaol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The shogaol mixture so obtained was subjected to reverse phase column chromatography to obtain 6-shogaol. The purity and identity of 6-shogaol was then verified using ^{1}H NMR ($CDCl_3$, 400 Hz).

FIGURE 15

^{13}C NMR of 6-shogaol



Ginger extract was subjected to solvent extraction using methanol, ethyl acetate and hexane. The methanolic portion was then evaporated and subjected to sephadex column chromatography. The resulting fractions were combined, subjected to diaon HP-20 column chromatography and eluted with 30-70% ethanol. Similar fractions were combined. The shogaol mixture so obtained was subjected to reverse phase column chromatography to obtain 6-shogaol. The purity and identity of 6-shogaol was then verified using ^{13}C NMR ($CDCl_3$, 100 Hz).

II. Evaluation of anti-inflammatory activities of gingerols and shogaols using the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear inflammatory model

Effect of 6-gingerol on TPA-induced ear inflammation in mice

In this preliminary study, we wanted to study the anti-inflammatory effects of 6-gingerol on TPA-induced ear inflammation. The effect of 6-gingerol (up to a dose of 1 μMol) on TPA-induced ear edema and over expressions of interleukins was evaluated as described previously. 6-gingerol decreased ear edema induced by TPA in a dose dependent manner significantly as shown in Table 2. However only the highest dose of 6-gingerol (1μmol) was able to inhibit the levels of the cytokines as shown in Table 2. Hence in our second experiment we have used higher concentrations of 6-gingerol and 6-shogaol to evaluate their anti-inflammatory activities using the same model.

TABLE 2

Effect of 6-gingerol on TPA-induced ear inflammation in CD-1 mice

Treatment	Weight of ear punch (mg)	IL-1β concentration (pg/mg tissue)	IL-6 concentration (pg/mg tissue)
1. Acetone	7.32 ± 0.35	2.24 ± 0.07	0.87 ± 0.01
2. TPA (1.5 nmol)	16.72 ± 0.56	22.78 ± 1.46	1.99 ± 0.44
3. 6-gingerol (0.25 μmol) + TPA (1.5 nmol)	14.86 ± 0.99* (20%)	24.50 ± 0.71	3.25 ± 0.30
4. 6-gingerol (0.5 μmol) + TPA (1.5 nmol)	13.82 ± 1.38* (31%)	21.58 ± 1.65	2.91 ± 0.53
5. 6-gingerol (1 μmol) + TPA (1.5 nmol)	13.59 ± 1.40* (33%)	18.42 ± 0.34* (16%)	1.7 ± 0.12* (26%)

Female CD-1 mice (7 weeks old, 5 mice/group) were treated topically with acetone or test compound in acetone 20 minutes prior to application of acetone or TPA (1.5 nmol) in acetone. The mice were sacrificed 6 hours after TPA treatment and ears punched. The ear punches were then weighed, grouped and homogenized in PBS. The homogenate was used for the quantification of IL-1β and IL-6. The data are expressed as the mean ± standard deviation from three separate values. Values with asterix (*) are significantly different ($p<0.05$) from the positive control (TPA) group, as determined by the Student *t* test. Values in parenthesis indicate the percentage inhibition compared to the positive control group.

Effect of 6-gingerol and 6-shogaol on TPA-induced ear inflammation in mice

In this study, the effect of 6-gingerol and 6-shogaol (at 25 and 50 μmol) on TPA-induced ear edema and over expressions of interleukins was evaluated. Briefly, both ears of female CD-1 mice were treated with acetone or test compounds prior to application of TPA. At the end of treatment the mice were sacrificed and ear punches weighed. Ear punches from each group was then combined and homogenized in PBS. This homogenate was used for the determination of IL-1β and 6 using ELISA. As shown previously, TPA induced edema as well as over expression of IL-1β and IL-6 in mouse ears. 6-gingerol and 6-shogaol were able to edema as well as levels of the inflammatory mediators significantly in a dose dependent manner as shown in Table 3. 6-shogaol was more effective than 6-gingerol in inhibiting all the markers of inflammation at the lower dose of 25 μMol.

TABLE 3

Effect of 6-gingerol and 6-shogaol on TPA-induced ear inflammation in CD-1 mice

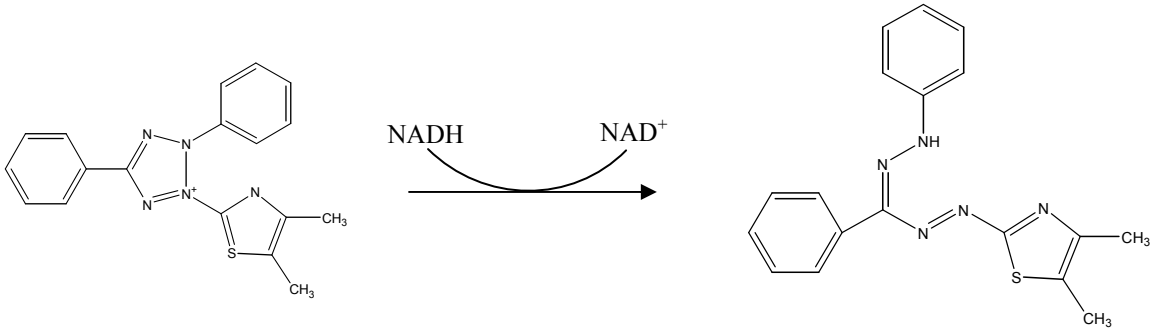
Treatment	Weight of Ear Punch (mg)	IL-1β concentration	IL-6 concentration
1. Acetone	6.89 ± 0.001	0.51 ± 0.09	1.90 ± 0.32
2. TPA (1.5 nmol)	14.53 ± 0.003	3.55 ± 0.06	4.85 ± 0.10
3. 6-gingerol (25 μmol) + TPA (1.5 nmol)	9.31 ± 0.002* (68%)	2.24 ± 0.25* (43%)	3.64 ± 0.16* (41%)
4. 6-gingerol (50 μmol) + TPA (1.5 nmol)	7.08 ± 0.001* (97%)	0.64 ± 0.06* (96%)	2.01 ± 0.26* (96%)
5. 6-shogaol (25 μmol) + TPA (1.5 nmol)	7.85 ± 0.001* (87%)	1.01 ± 0.09* (83%)	2.39 ± 0.28* (83%)
6. 6-shogaol (50 μmol) + TPA (1.5 nmol)	6.83 ± 0.001* (100%)	0.75 ± 0.17* (92%)	2.03 ± 0.28* (96%)

Female CD-1 mice (7 weeks old, 5 mice/group) were treated topically with acetone or test compound in acetone 20 minutes prior to application of acetone or TPA (1.5 nmol) in acetone. The mice were sacrificed 6 hours after TPA treatment and ears punched. The ear punches were then weighed, grouped and homogenized in PBS. The homogenate was used for the quantification of IL-1β and IL-6. The data are expressed as the mean ± standard deviation from three separate values. Values with asterix (*) are significantly different ($p<0.01$) from the positive control (TPA) group, as determined by the Student *t* test. Values in parenthesis indicate percentage inhibition compared to the positive control group.

III. Evaluation of chemopreventive activities of gingerols and shogaols on prostate cancer cells (LNCaP and PC-3)

Effect of gingerol mixture, 6-gingerol, 8-gingerol, shogaol mixture and 6-shogaol on viability of LNCaP and PC-3 cells measured using MTT assay

Effect of the test compounds on cell viability was evaluated using the MTT assay as described previously. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow colored tetrazol product that is reduced to the purple colored formazan product as shown below in the mitochondria of viable cells using the NADH enzyme system.

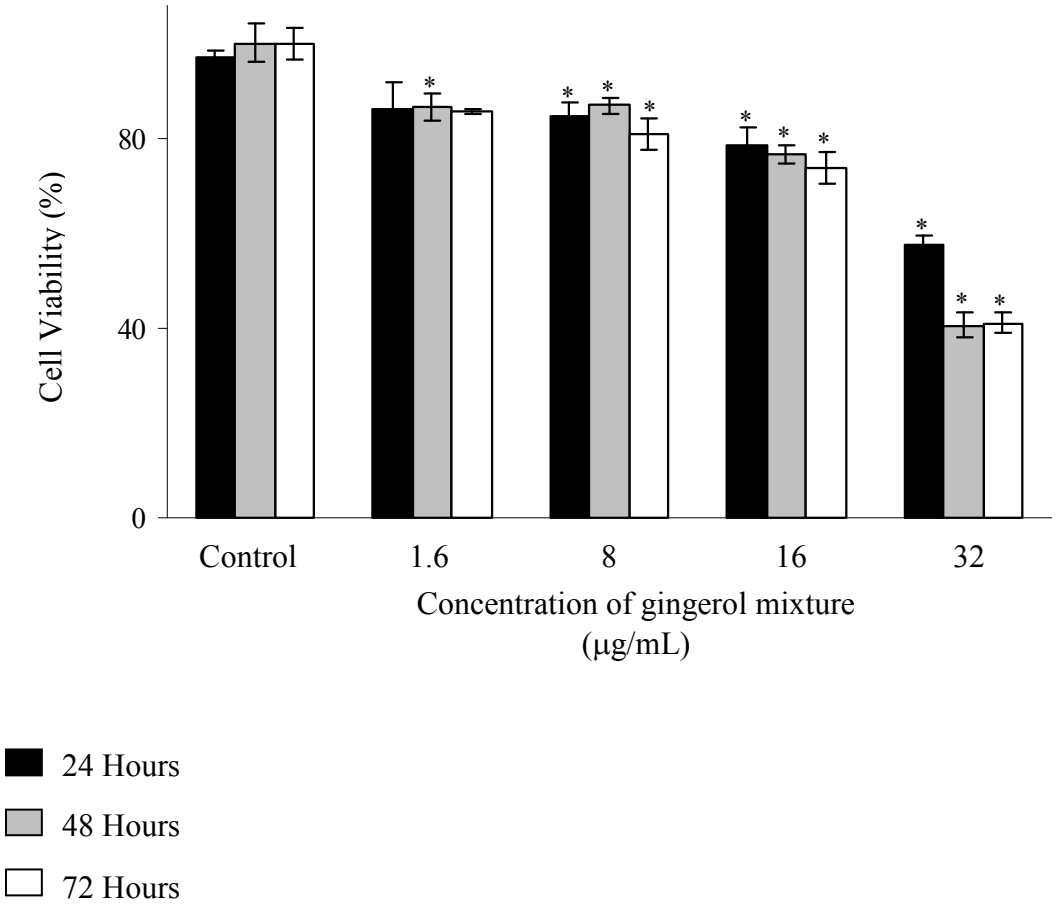


MTT Formazan

This assay is commonly used to assess cell proliferation and cytotoxicity of potential chemopreventive compounds. Gingerols and shogaols inhibited the growth of LNCaP and PC-3 cells in a dose and time dependent manner, as shown in Figures 16-25. Among, the test compounds, 6-shogaol and 8-gingerol were the most potent in inhibiting cell viability.

FIGURE 16

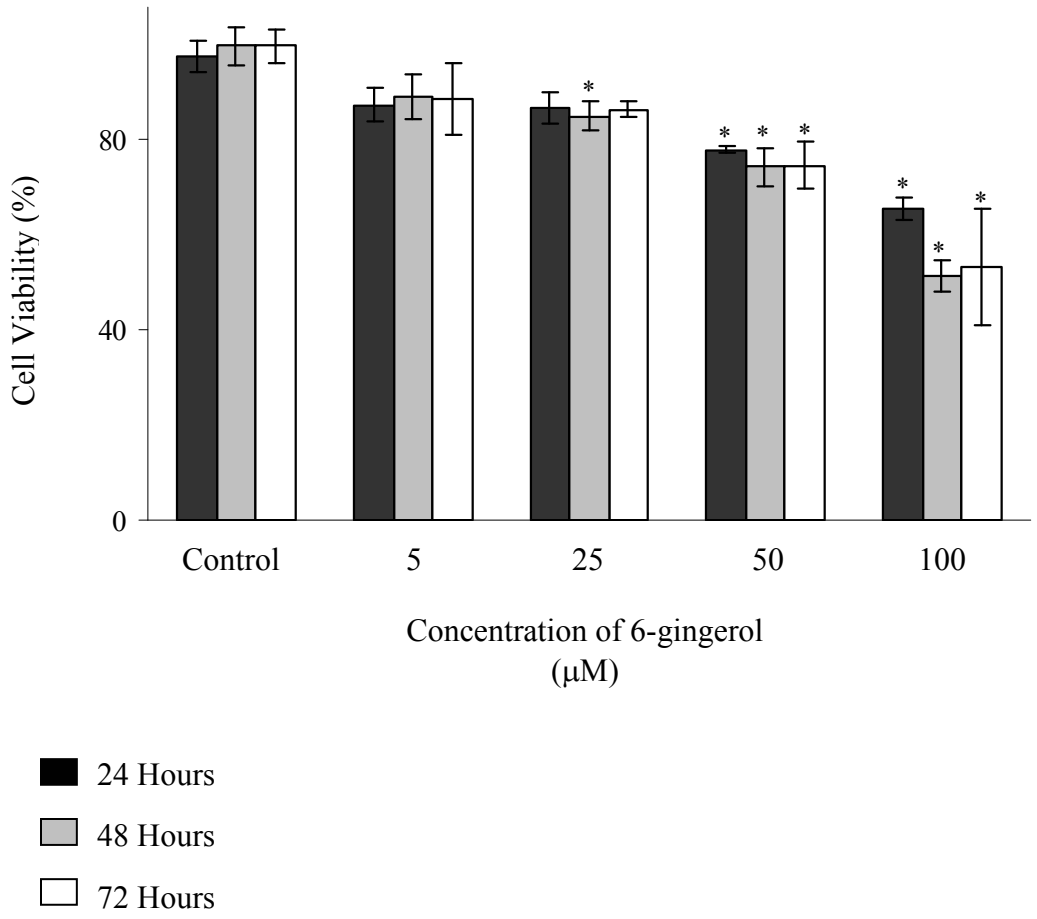
Effect of gingerol mixture on viability of LNCaP cells



LNCaP cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean ± standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 17

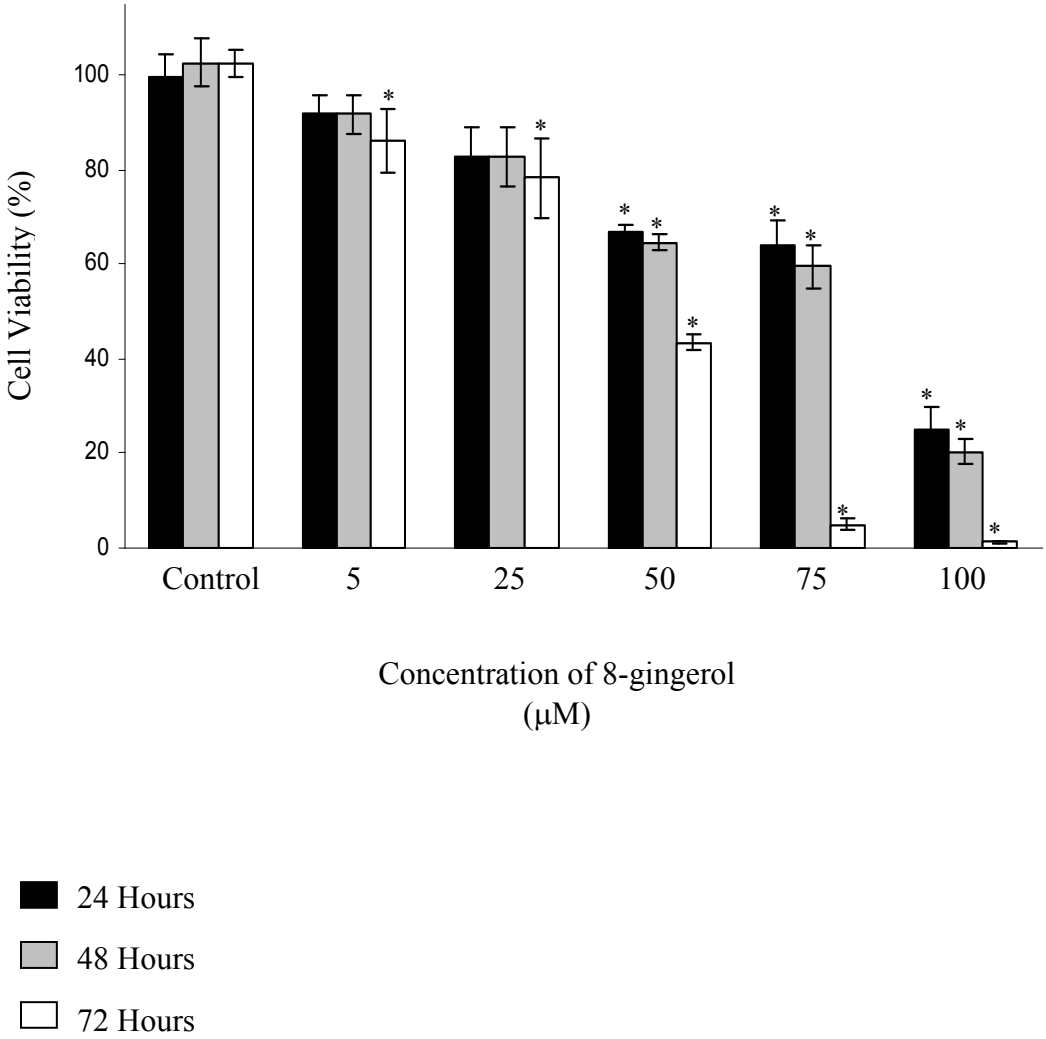
Effect of 6-gingerol on viability of LNCaP cells



LNCaP cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 18

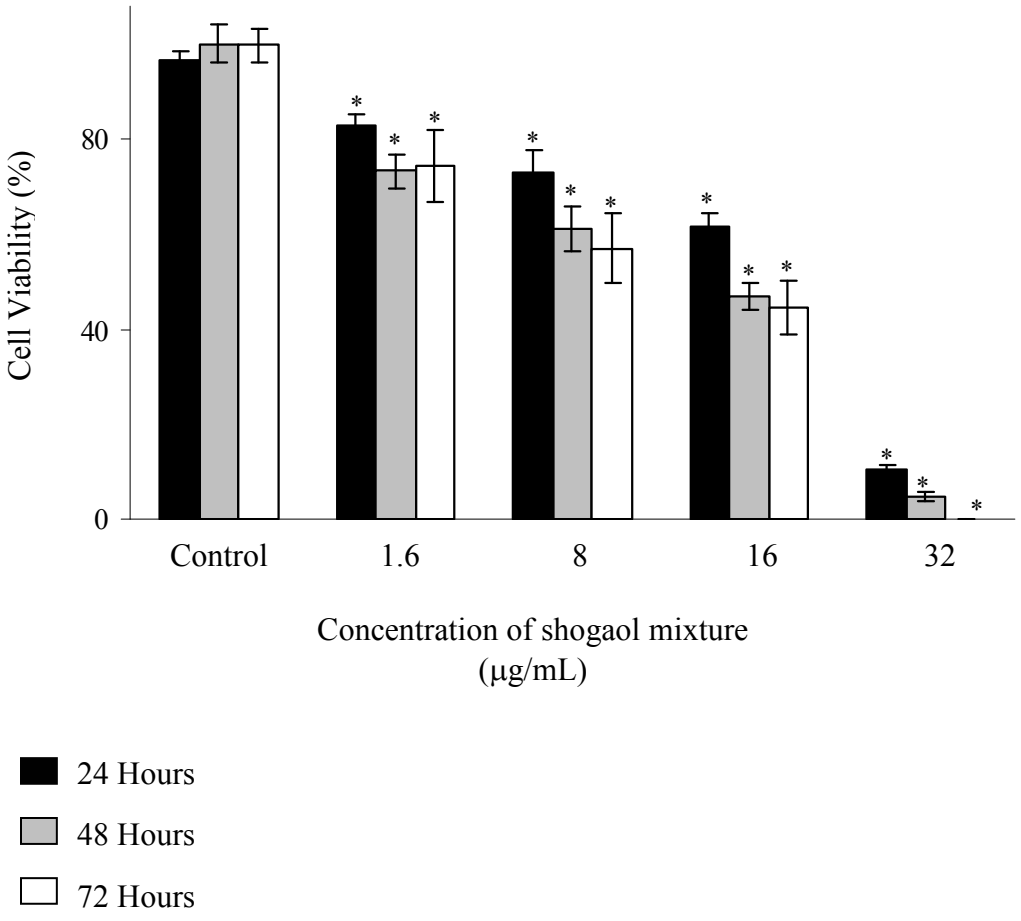
Effect of 8-gingerol on viability of LNCaP cells



LNCaP cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 19

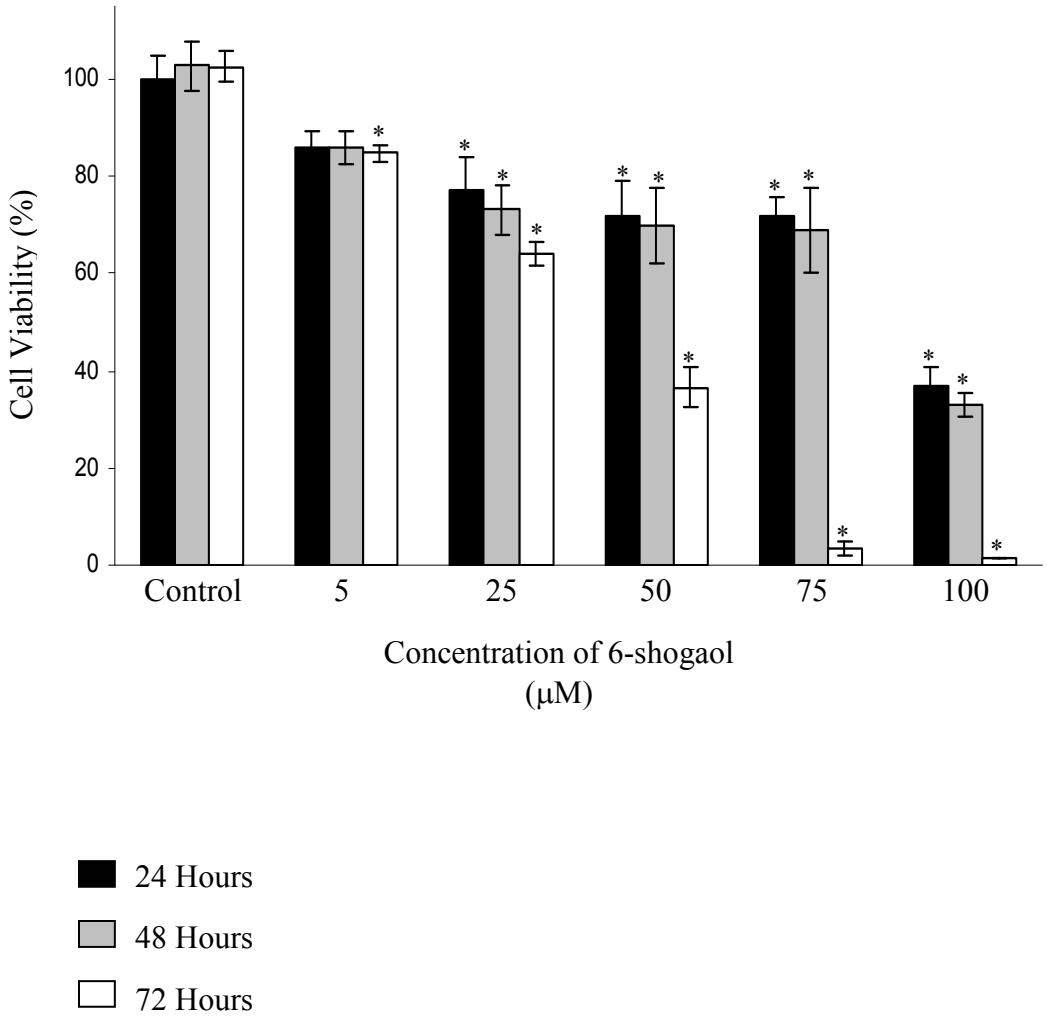
Effect of shogaol mixture on viability of LNCaP cells



LNCaP cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 20

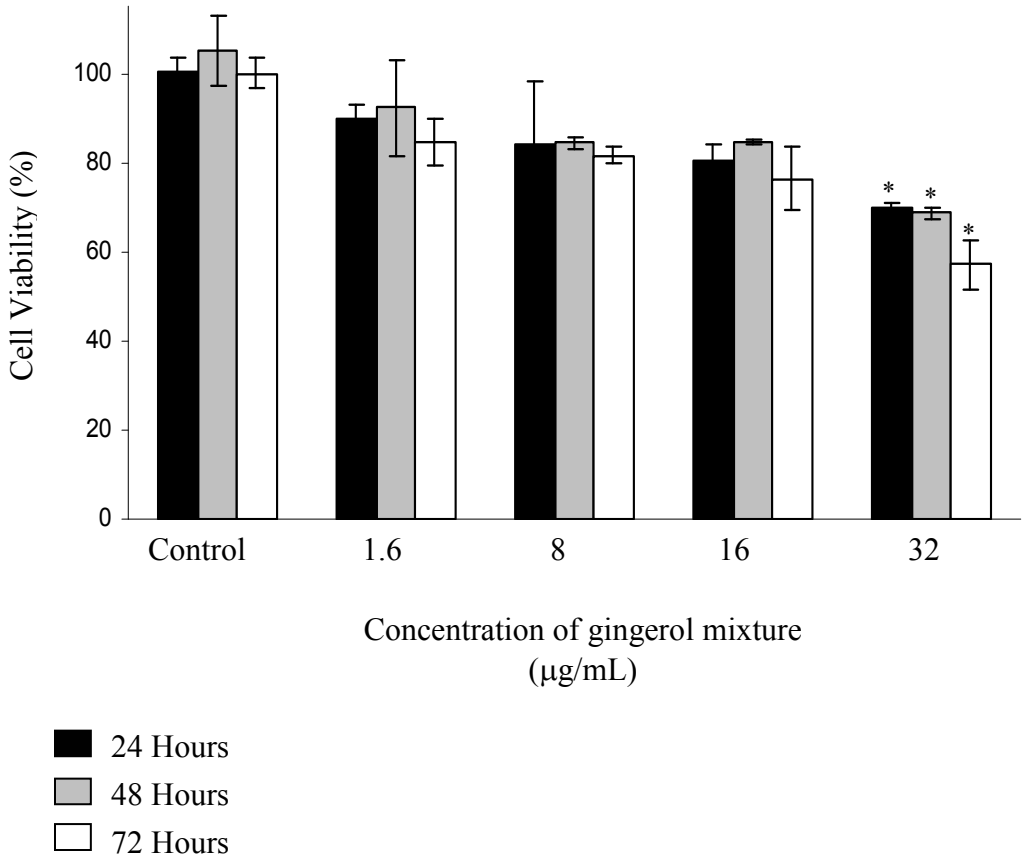
Effect of 6-shogaol on viability of LNCaP cells



LNCaP cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 21

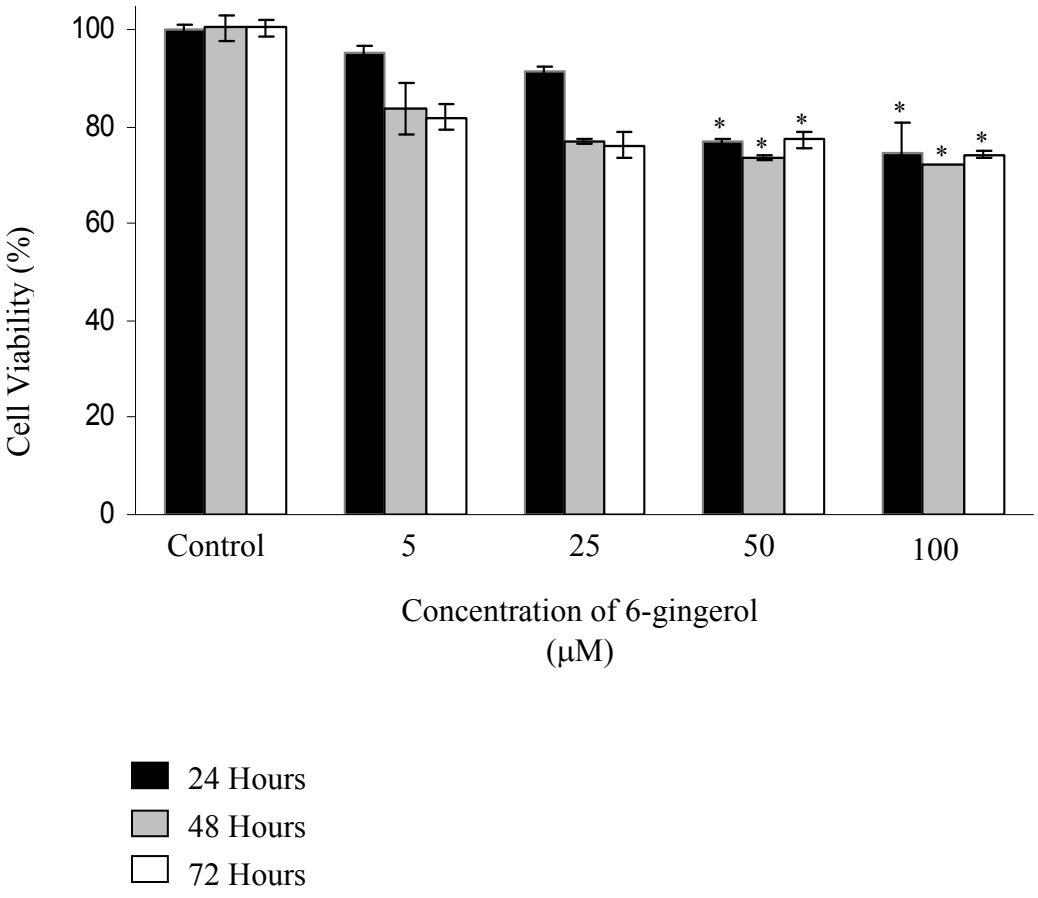
Effect of gingerol mixture on viability of PC-3 cells



PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 22

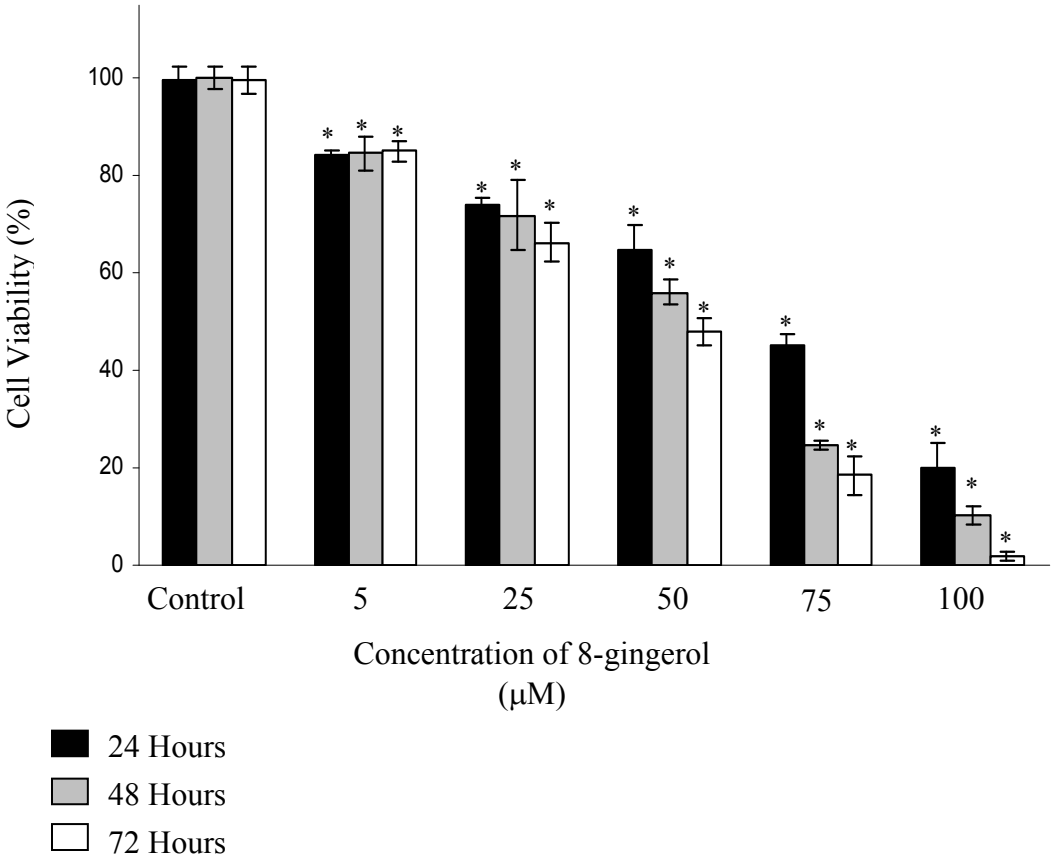
Effect of 6-gingerol on viability of PC-3 cells



PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 23

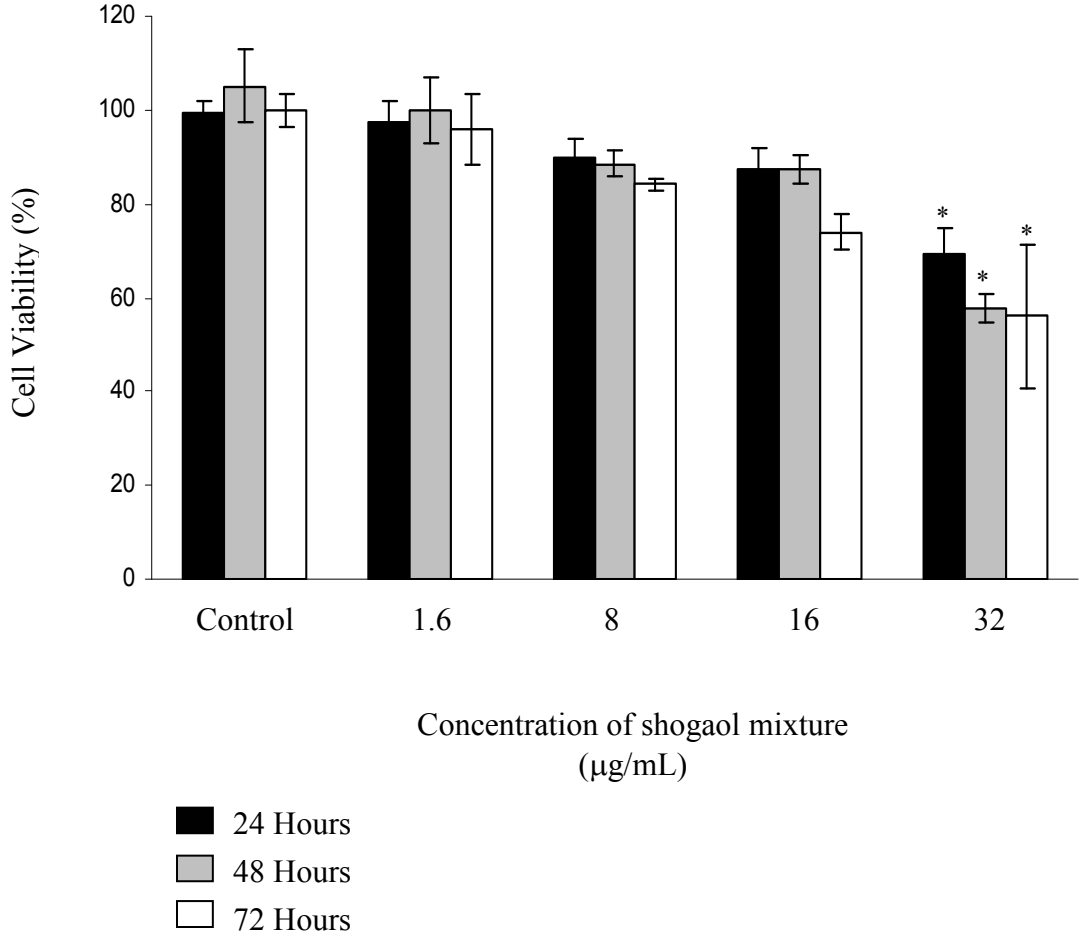
Effect of 8-gingerol on viability of PC-3 cells



PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 24

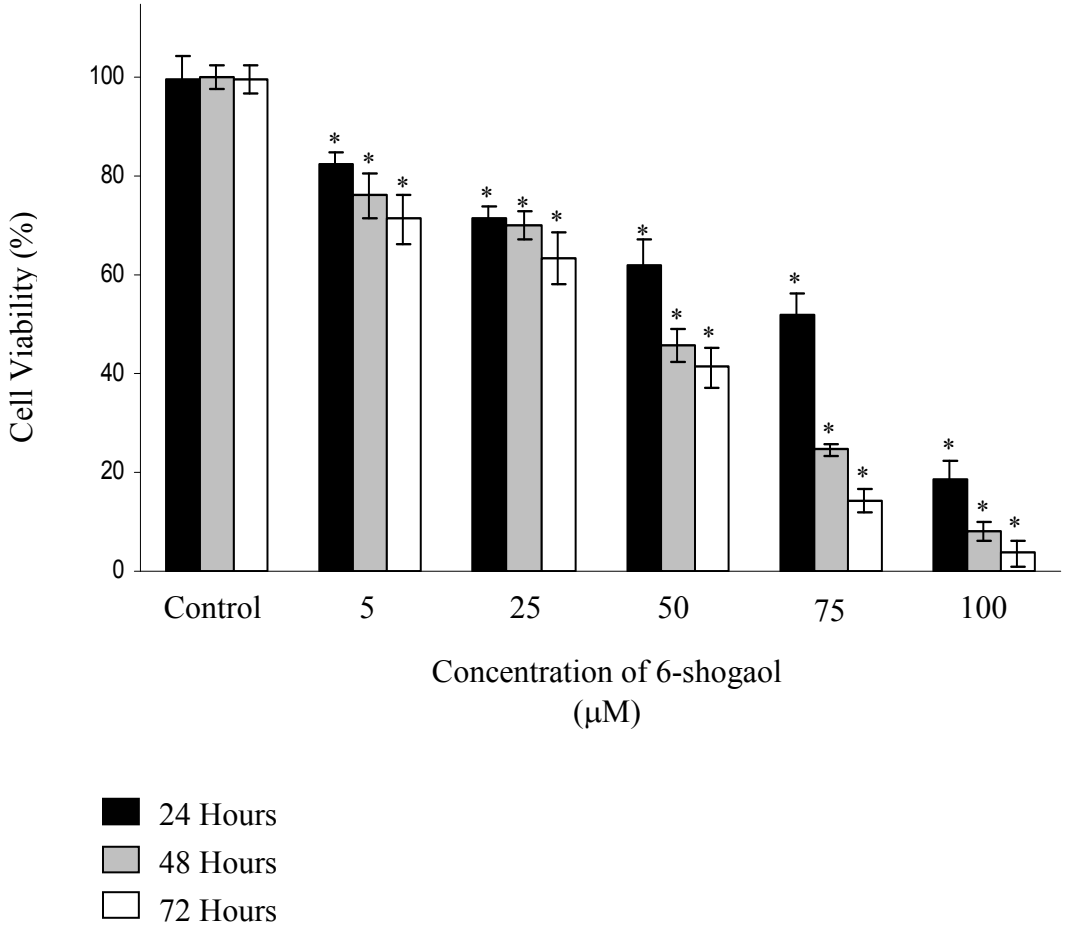
Effect of shogaol mixture on viability of PC-3 cells



PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

FIGURE 25

Effect of 6-shogaol on viability of PC-3 cells



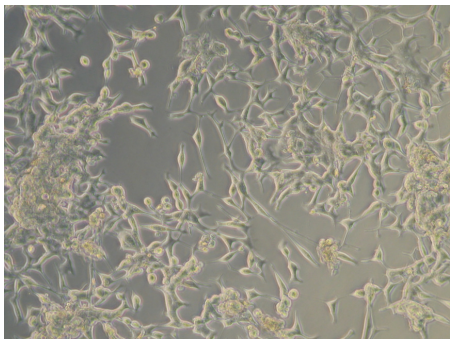
PC-3 cells were plated at a density of 1x 10^4 cells/well in 96 well plates and incubated at 37 °C, 5% carbon dioxide and 95% humidity atmosphere for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 24, 48 and 72 hours. At the end of the treatment, the cells were treated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4-5 hours. The formazan product was solubilized with DMSO and the absorbance measured at 570 nm. The data is expressed as the mean $\pm$ standard error of cell viability from six separate determinations. Values with asterix (*) are significantly different from the control group as measured using the Students *t* test.

Effect of the test compounds on LNCaP and PC-3 cell morphology

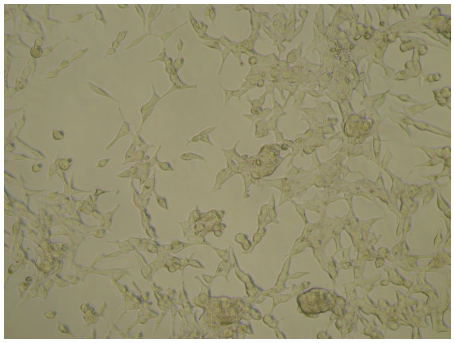
The effect of gingerol mixture, 6-gingerol, 8-gingerol, shogaol mixture and 6-shogaol on LNCaP and PC-3 cell morphology was also evaluated. LNCaP and PC-3 cells were plated at a density of 5 x 10^4 cells per well in 24 well tissue culture dishes and incubated for 24 hours. The cells were then treated with different concentrations of the test compounds and incubated for 48 hours. The cells were then viewed under an inverted microscope at 100x to observe the morphology of the cells. The results are shown in Figures 26-35. Shogaol mixture, 6-shogaol and 8-gingerol were most effective in inhibiting cell growth and inducing morphological changes in both LNCaP and PC-3 cells.

FIGURE 26

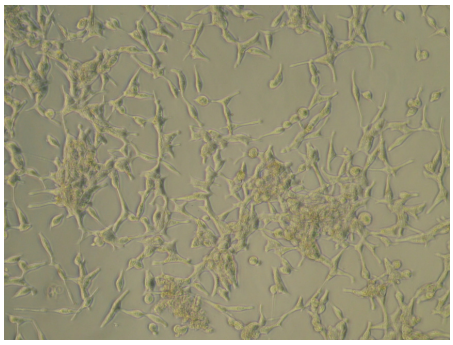
Effect of gingerol mixture on morphology of LNCaP cells



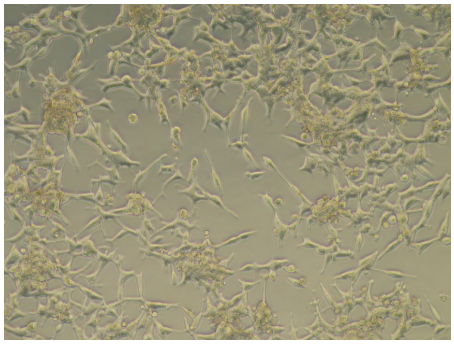
Control



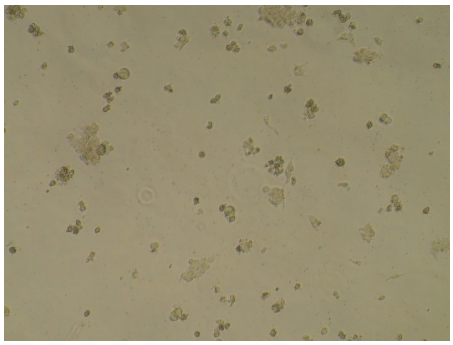
1.6 μg/mL gingerol mixture



8 μg/mL gingerol mixture



16 μg/mL gingerol mixture

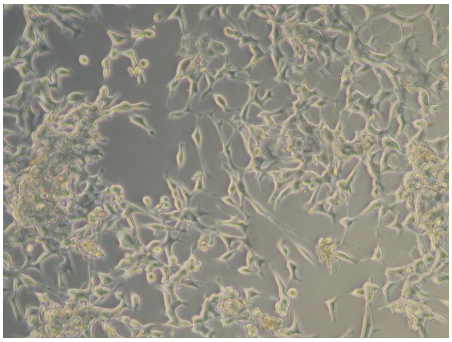


32 μg/mL gingerol mixture

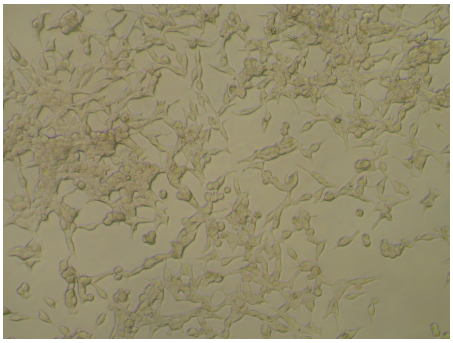
LNCaP cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 27

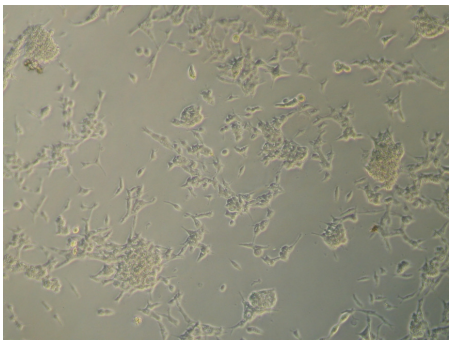
Effect of 6-gingerol on morphology of LNCaP cells



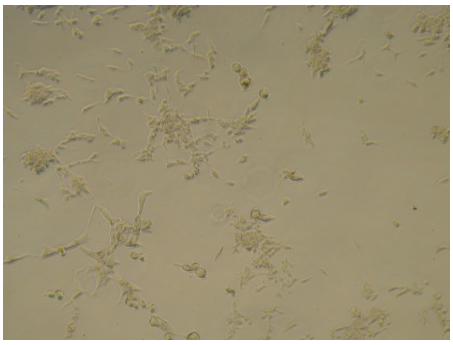
Control



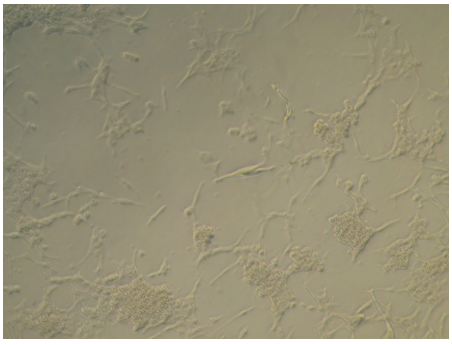
5 μM 6-gingerol



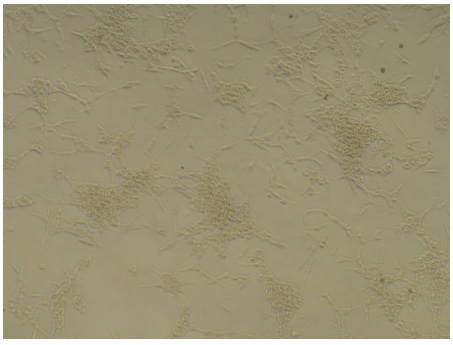
25 μM 6-gingerol



50 μM 6-gingerol



75 μM 6-gingerol

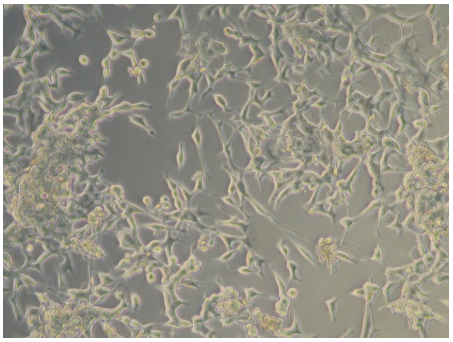


100 μM 6-gingerol

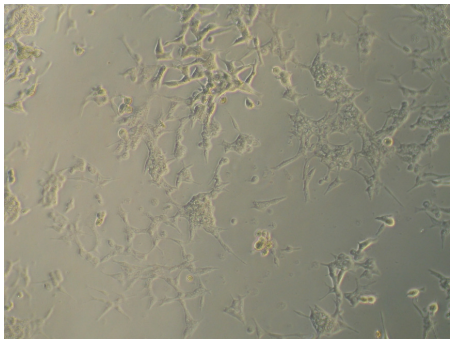
LNCaP cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 28

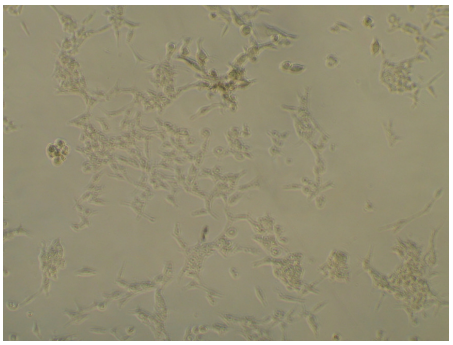
Effect of 8-gingerol on morphology of LNCaP cells



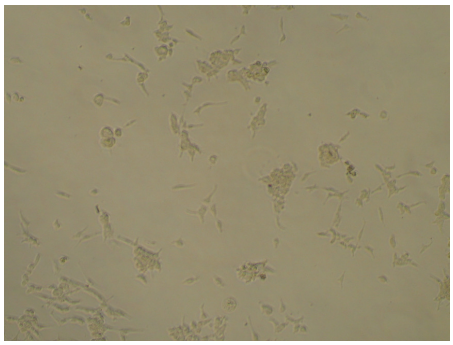
Control



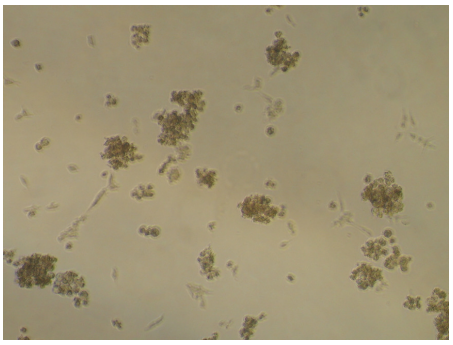
5 μM 8-gingerol



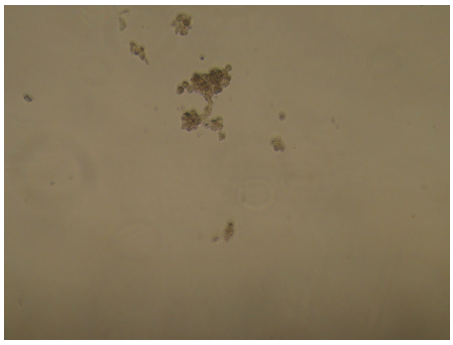
25 μM 8-gingerol



50 μM 8-gingerol



75 μM 8-gingerol

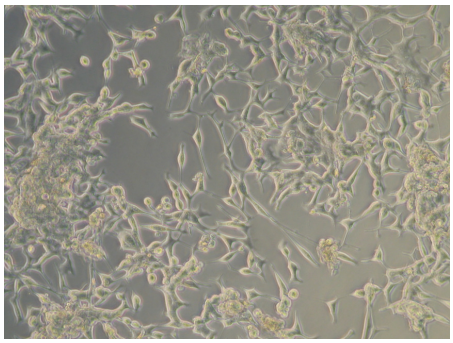


100 μM 8-gingerol

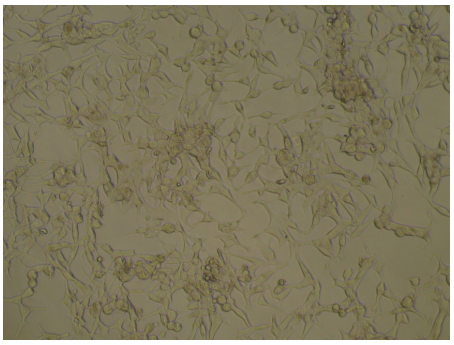
LNCaP cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 29

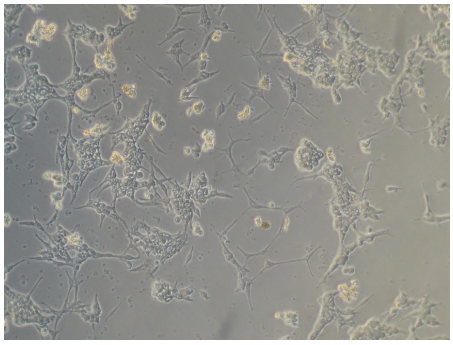
Effect of shogaol mixture on morphology of LNCaP cells



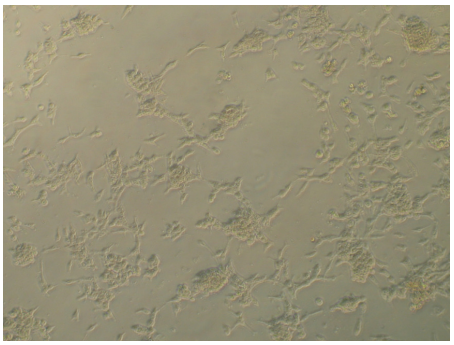
Control



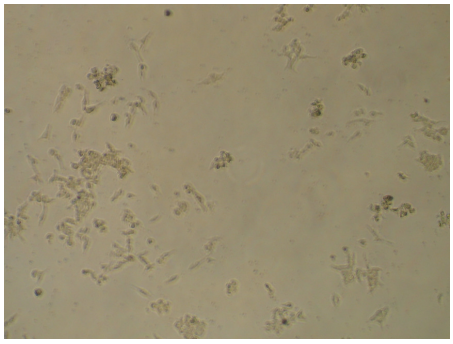
1.6 μg/mL shogaol mixture



8 μg/mL shogaol mixture



16 μg/mL shogaol mixture

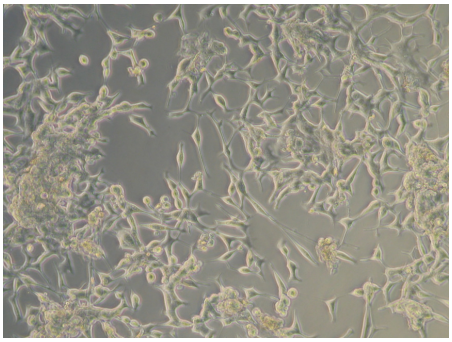


32 μg/mL shogaol mixture

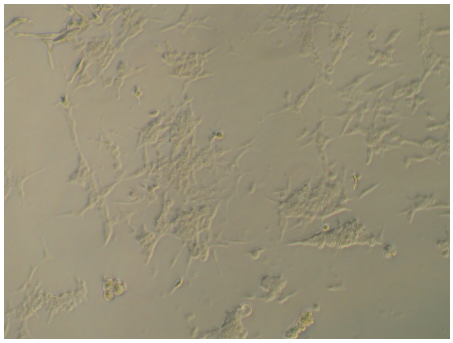
LNCaP cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 30

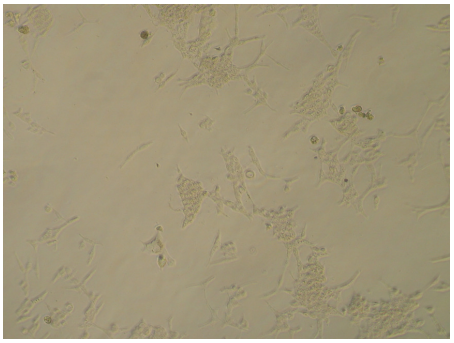
Effect of 6-shogaol on morphology of LNCaP cells



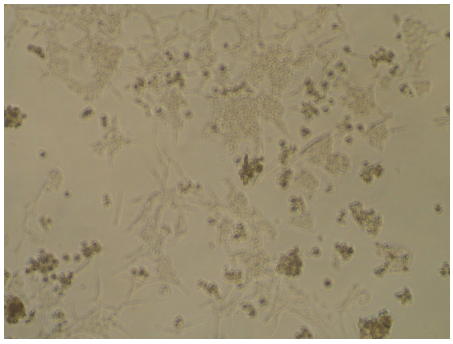
Control



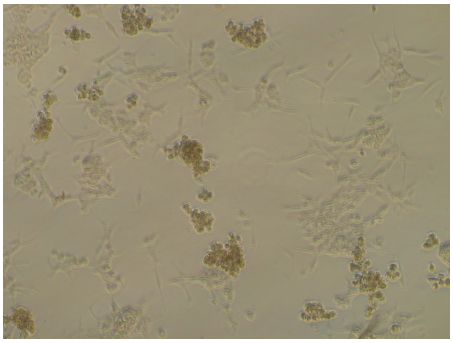
5 μM 6-shogaol



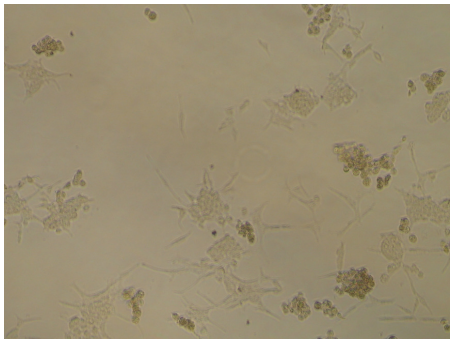
25 μM 6-shogaol



50 μM 6-shogaol



75 μM 6-shogaol

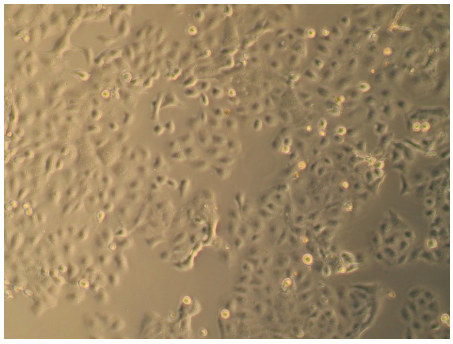


100 μM 6-shogaol

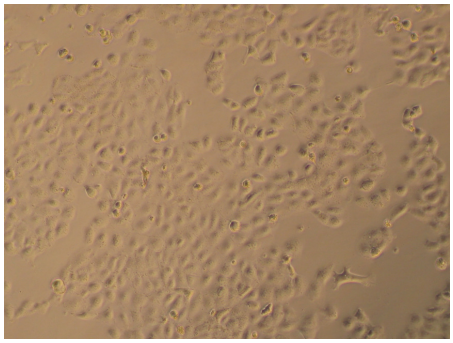
LNCaP cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 31

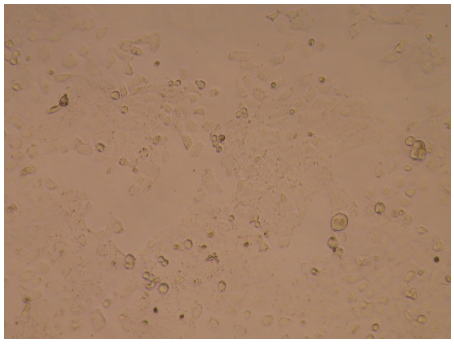
Effect of gingerol mixture on morphology of PC-3 cells



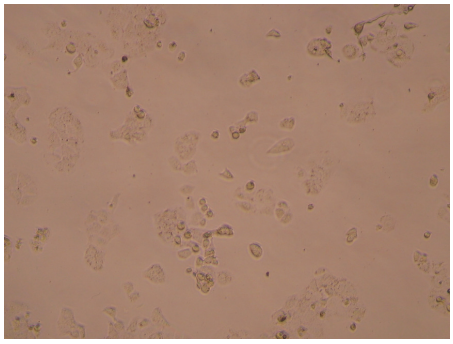
Control



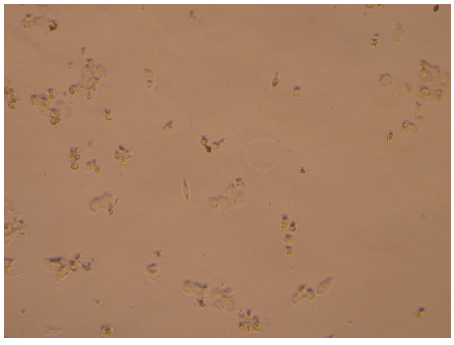
1.6 μg/mL gingerol mixture



8 μg/mL gingerol mixture



16 μg/mL gingerol mixture

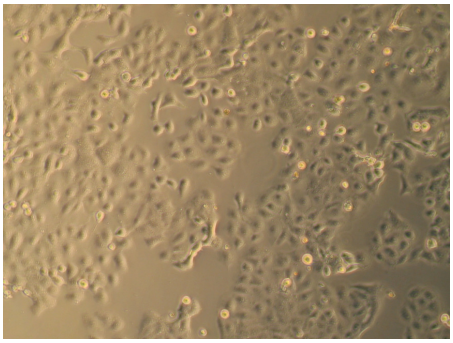


32 μg/mL gingerol mixture

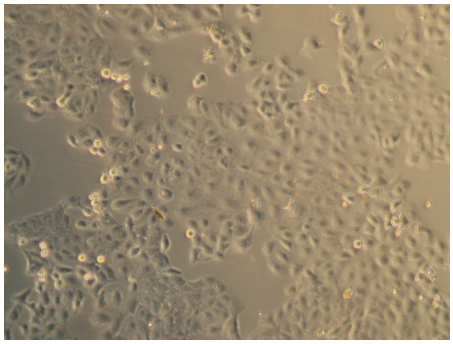
PC-3 cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 32

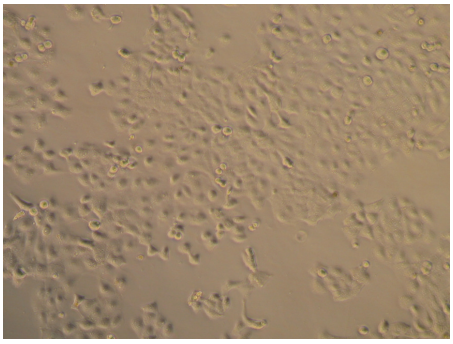
Effect of 6-gingerol on morphology of PC-3 cells



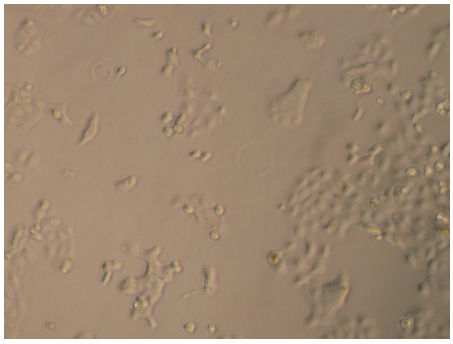
Control



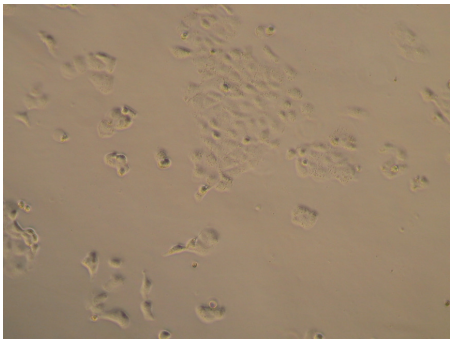
5 μM 6-gingerol



25 μM 6-gingerol



50 μM 6-gingerol

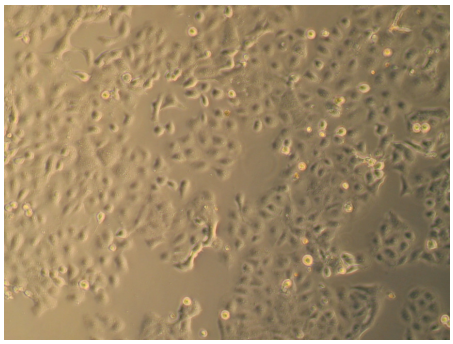


100 μM 6-gingerol

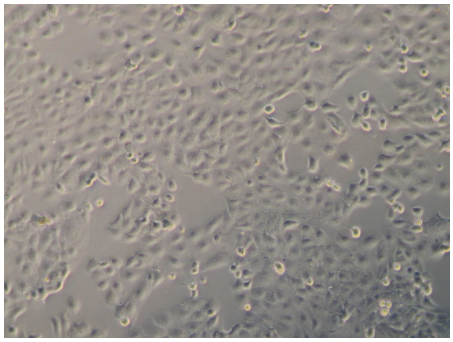
PC-3 cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 33

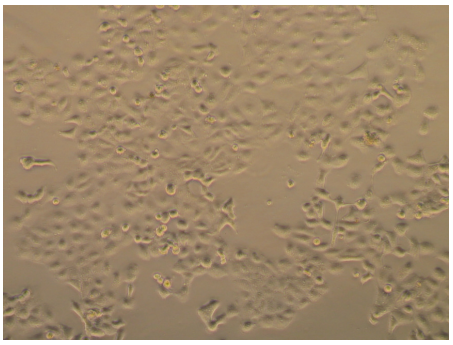
Effect of 8-gingerol on morphology of PC-3 cells



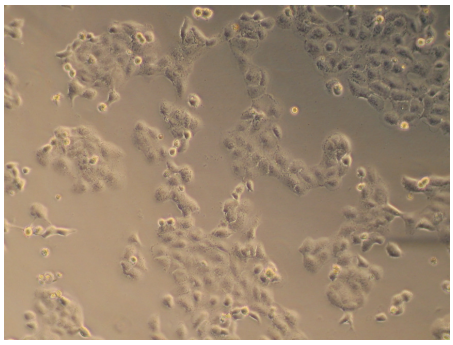
Control



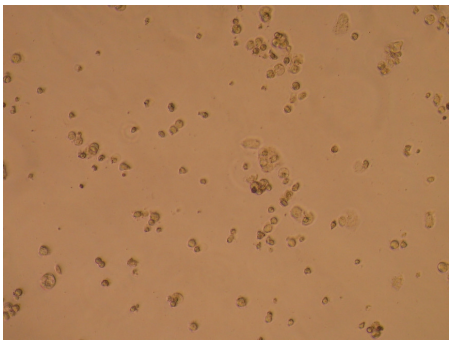
5 μM 8-gingerol



25 μM 8-gingerol



50 μM 8-gingerol

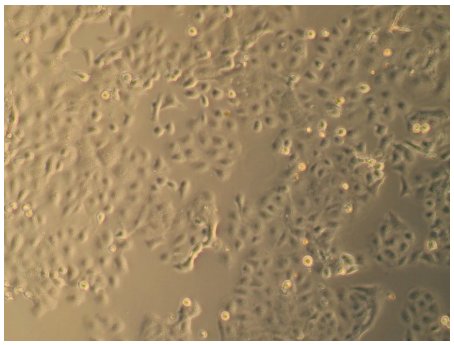


100 μM 8-gingerol

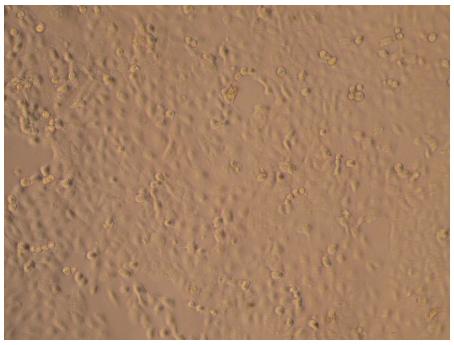
PC-3 cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 34

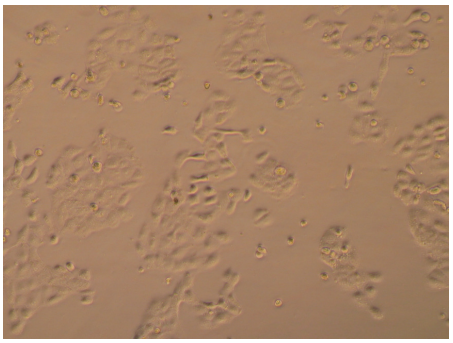
Effect shogaol mixture on morphology of PC-3 cells



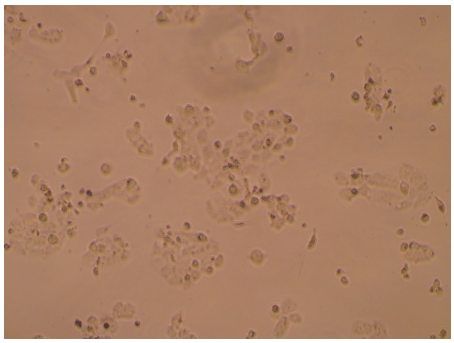
Control



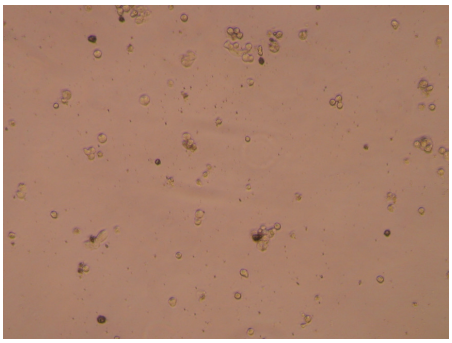
1.6 μg/mL shogaol mixture



8 μg/mL shogaol mixture



16 μg/mL shogaol mixture

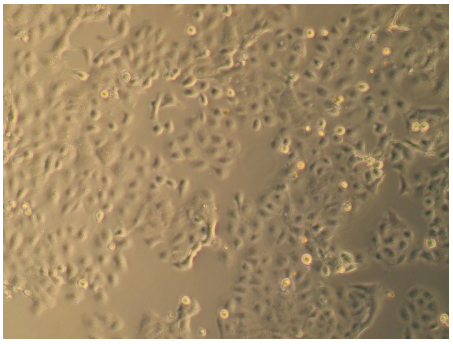


32 μg/mL shogaol mixture

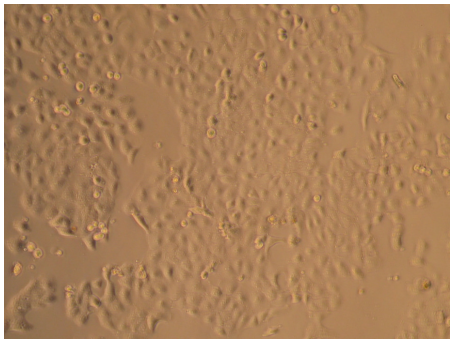
PC-3 cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

FIGURE 35

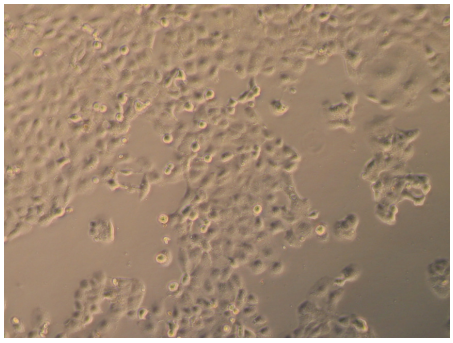
Effect 6-shogaol on morphology of PC-3 cells



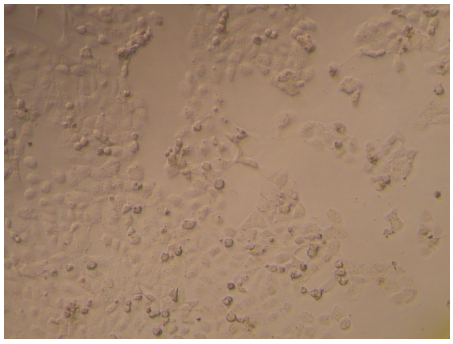
Control



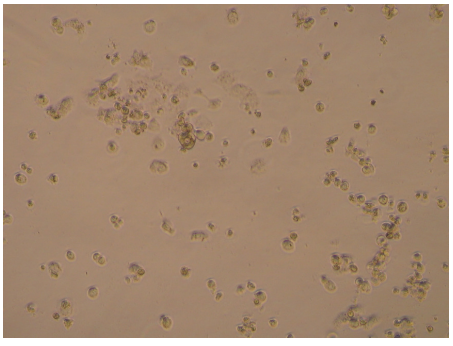
5 μM 6-shogaol



25 μM 6-shogaol



50 μM 6-shogaol



100 μM 6-shogaol

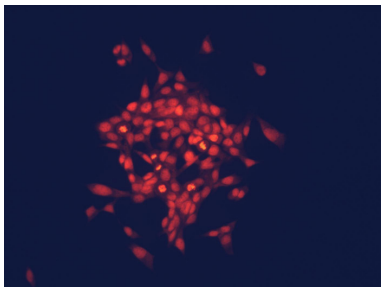
PC-3 cells were seeded at a density of $5x10^4$ cells/mL and incubated for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were viewed under an inverted microscope at 100x to observe the morphology.

Effect of gingerols and shogaols on apoptosis measured using fluorescence microscopy

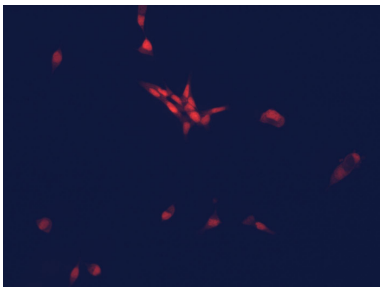
LNCaP and PC-3 cells were seeded at a density of 2 x 10^4 cells/mL in 6-well tissue culture plates (2 mL/well) and incubated at 37 °C, 5% CO_2 and 95% RH atmosphere for 24 hours. The cells were then treated with the test compound and incubated for an additional 48 hours. At the end of the treatment, the cells were fixed with a 1:1 mixture of acetone: methanol (1 mL/well) and stained with 1 mL of propidium iodide and viewed under the fluorescent microscope. Apoptotic cells were characterized by condensed and fragmented nuclei and the apoptotic ratio was determined using the formula: [(number of apoptotic cells/total number of cells) x 100]. All the test compounds induced apoptosis in both LNCaP and PC-3 cells as shown in Figures 36-44. 8-gingerol, shogaol mixture and 6-shogaol were the most effective in inducing apoptosis in both PC-3 and LNCaP cells, although the effect was stronger in the latter.

FIGURE 36 a

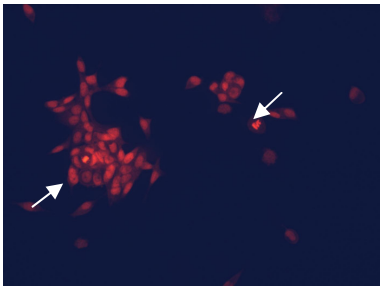
Effect of gingerol mixture on apoptosis in LNCaP cells



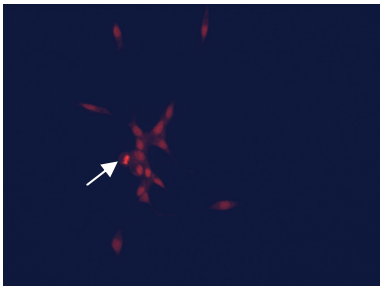
Control



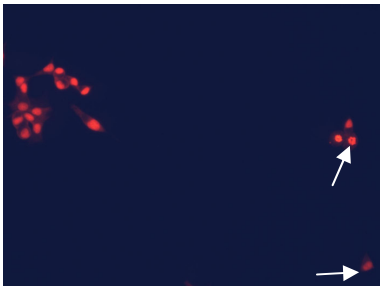
1.6 µg/mL gingerol mixture



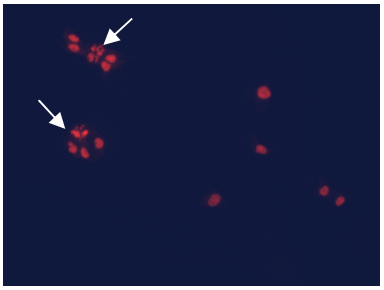
8 µg/mL gingerol mixture



16 µg/mL gingerol mixture



24 µg/mL gingerol mixture

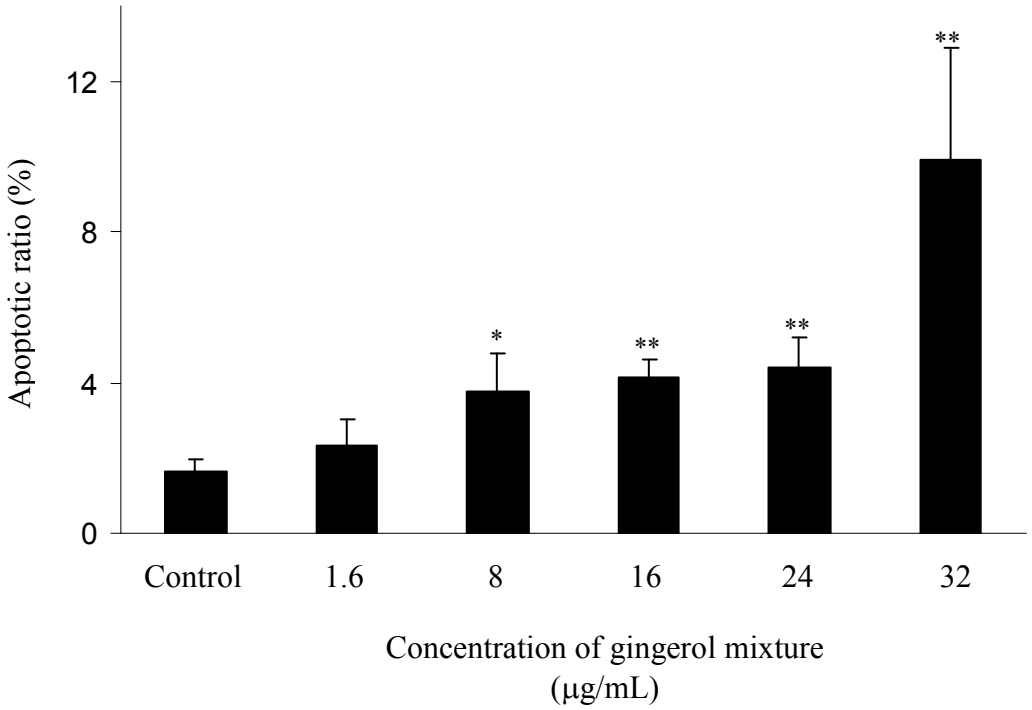


32 µg/mL gingerol mixture

LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1µg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 36 b

Effect of gingerol mixture on apoptosis in LNCaP cells

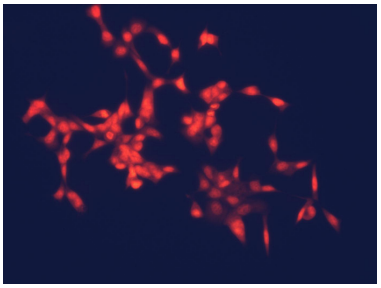


LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

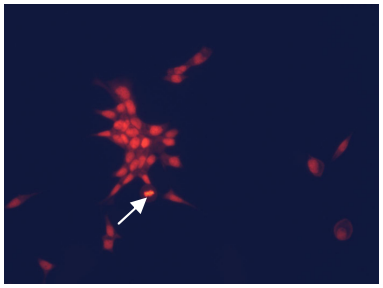
* $p<0.05$
** $p<0.01$

FIGURE 37 a

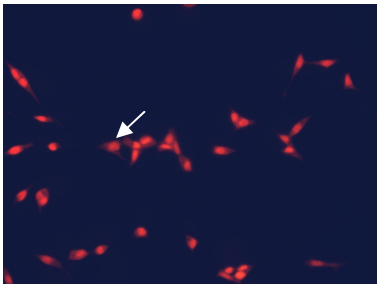
Effect of 6-gingerol on apoptosis in LNCaP cells



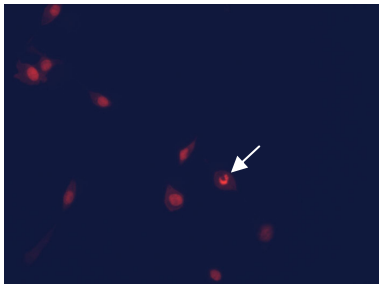
Control



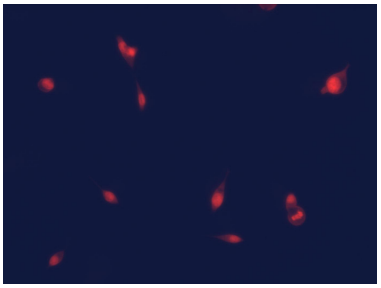
5 μM 6-Gingerol



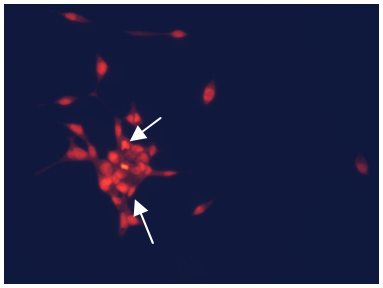
25 μM 6-Gingerol



50 μM 6-Gingerol



75 μM 6-Gingerol

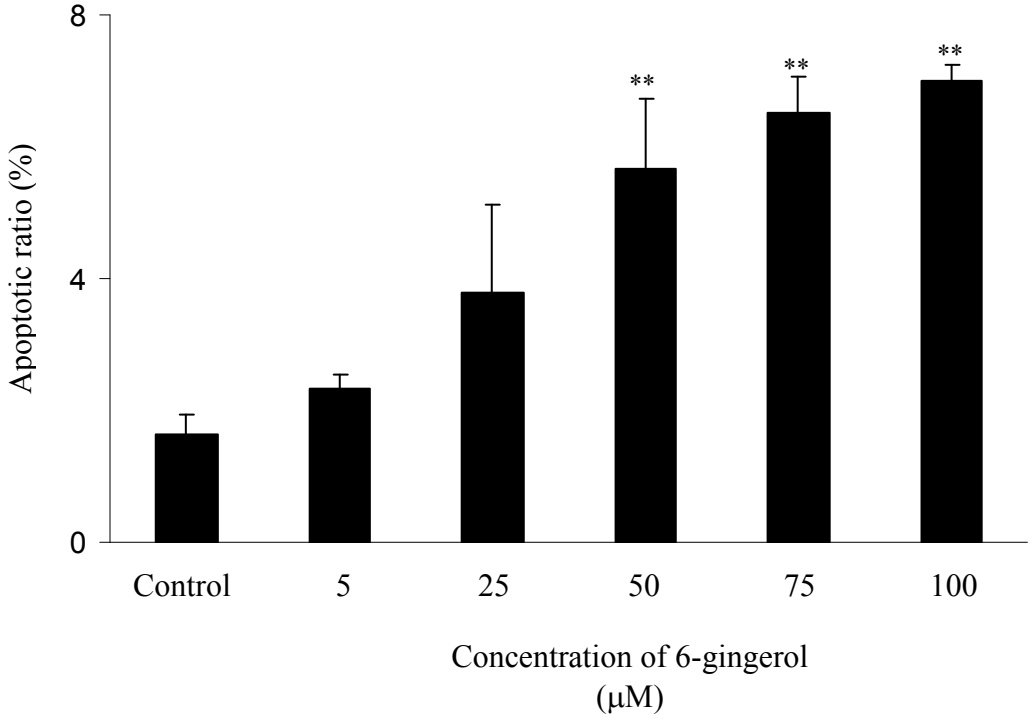


100 μM 6-Gingerol

LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 37 b

Effect of 6-gingerol on apoptosis in LNCaP cells

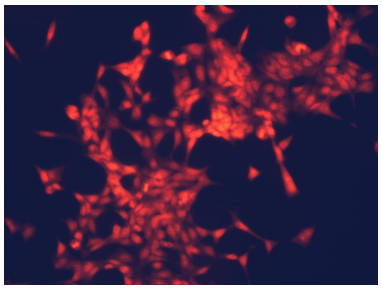


LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

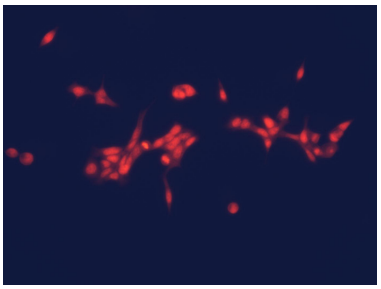
** $p<0.01$

FIGURE 38 a

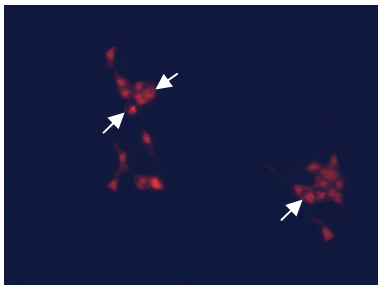
Effect of 8-gingerol on apoptosis in LNCaP cells



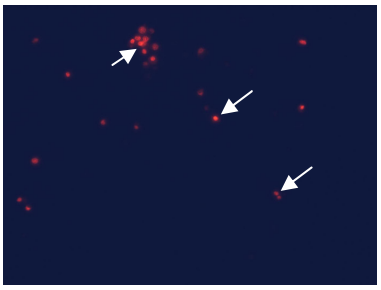
Control



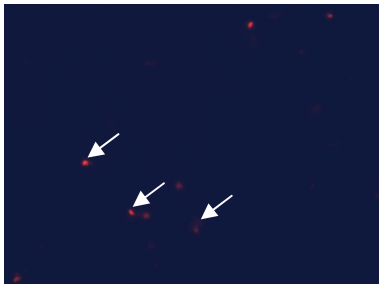
5 μM 8-Gingerol



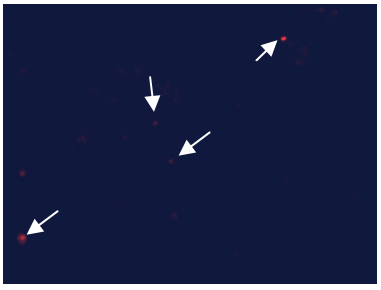
25 μM 8-Gingerol



50 μM 8-Gingerol



75 μM 8-Gingerol

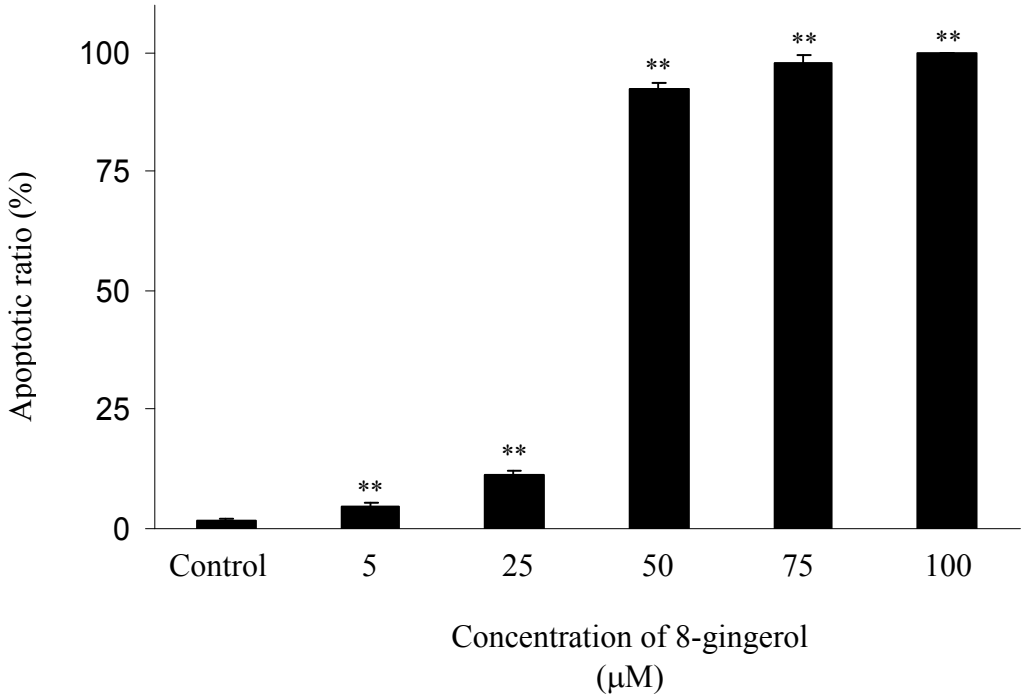


100 μM 8-Gingerol

LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 38 b

Effect of 8-gingerol on apoptosis in LNCaP cells

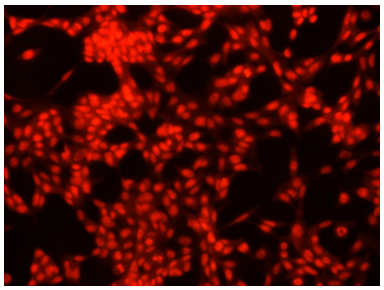


LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

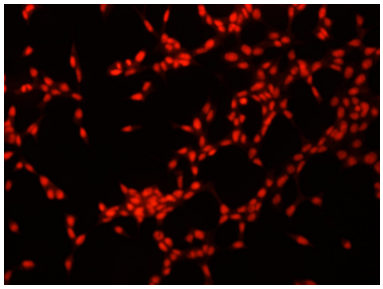
** $p<0.01$

FIGURE 39 a

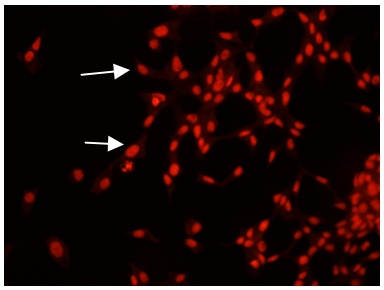
Effect of shogaol mixture on apoptosis in LNCaP cells



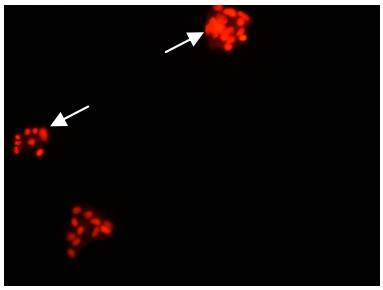
Control



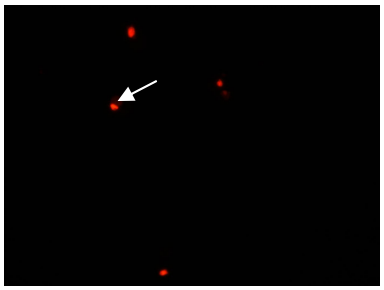
1.6 μg/mL shogaol mixture



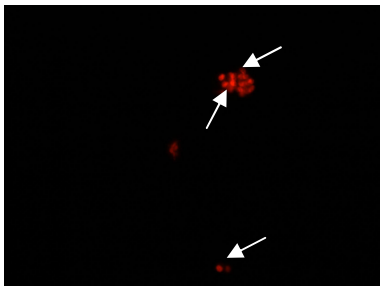
8 μg/mL shogaol mixture



16 μg/mL shogaol mixture



24 μg/mL shogaol mixture

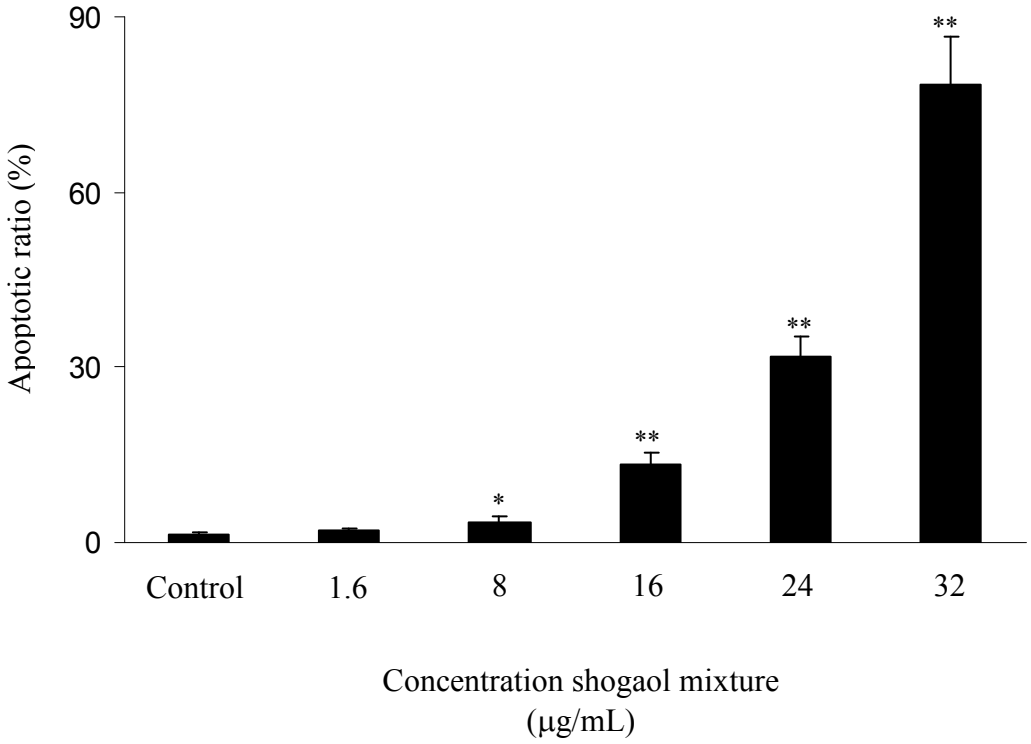


32 μg/mL shogaol mixture

LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 39 b

Effect of shogaol mixture on apoptosis in LNCaP cells

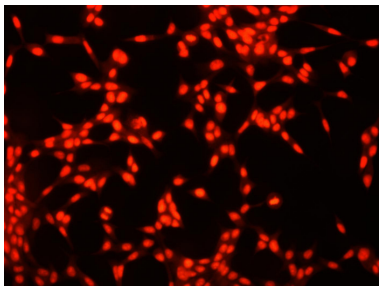


LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

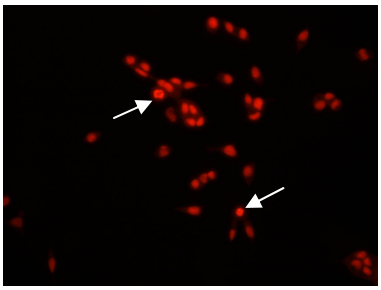
* $p<0.05$
** $p<0.01$

FIGURE 40 a

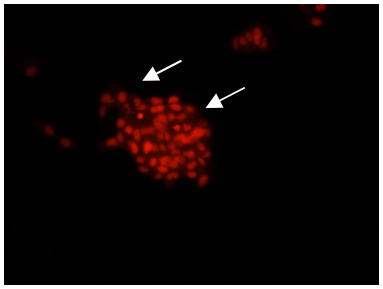
Effect of 6-shogaol on apoptosis in LNCaP cells



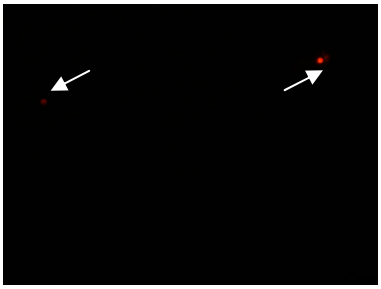
Control



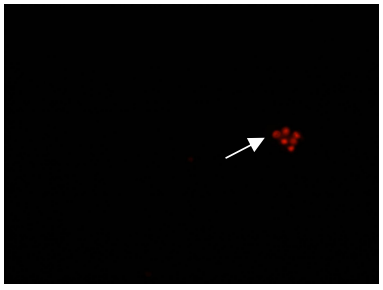
5 μM 6-shogaol



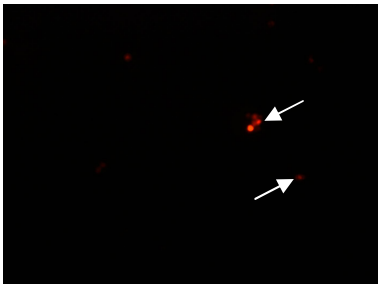
25 μM 6-shogaol



50 μM 6-shogaol



75 μM 6-shogaol

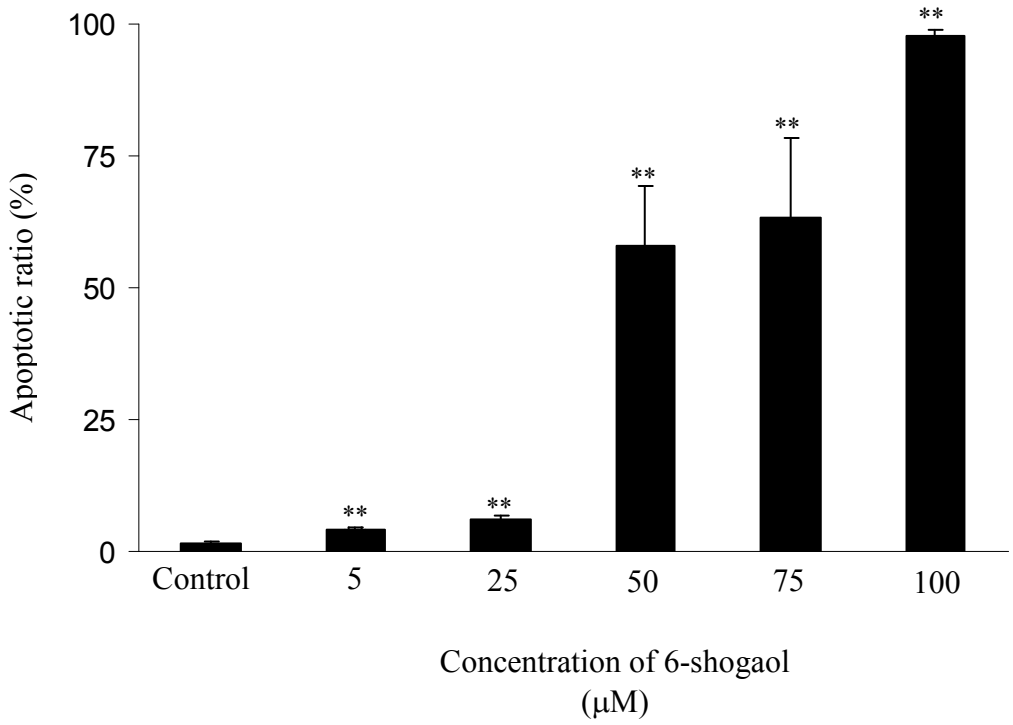


100 μM 6-shogaol

LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 40 b

Effect of 6-shogaol on apoptosis in LNCaP cells

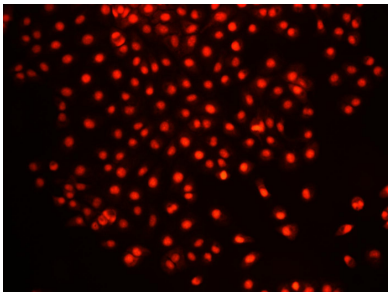


LNCaP cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

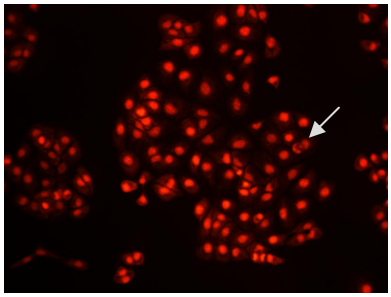
** $p<0.01$

FIGURE 41 a

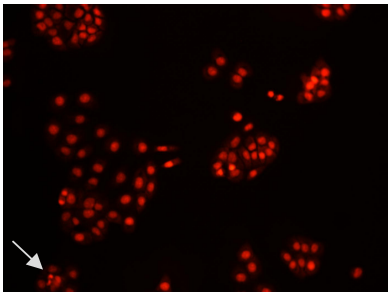
Effect of gingerol mixture on apoptosis in PC-3 cells



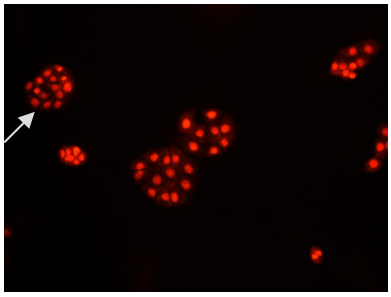
Control



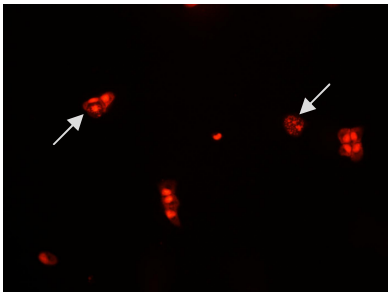
1.6 μg/mL gingerol mixture



8 μg/mL gingerol mixture



16 μg/mL gingerol mixture

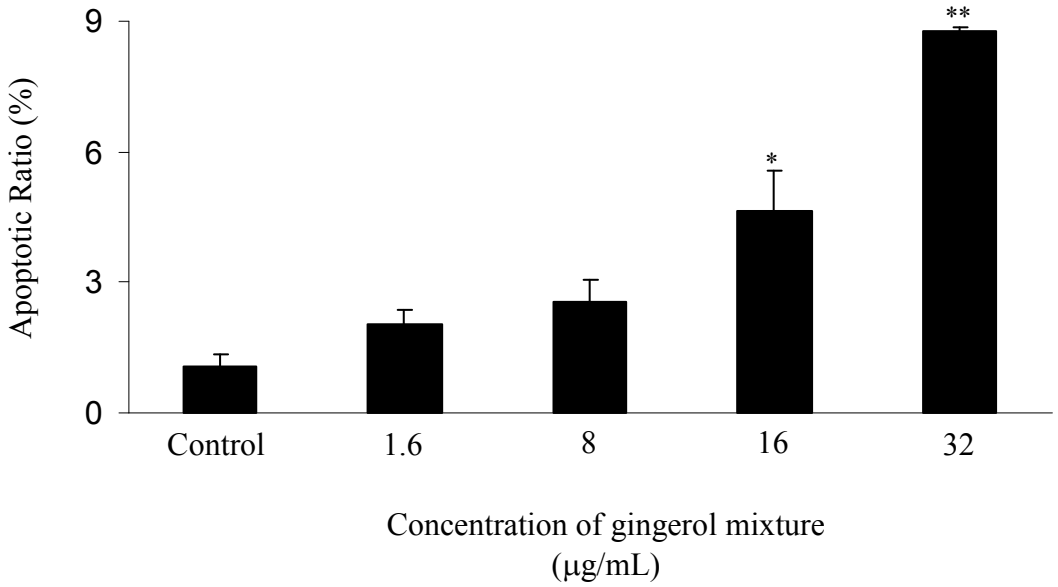


32 μg/mL gingerol mixture

PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 41 b

Effect of gingerol mixture on apoptosis in PC-3 cells

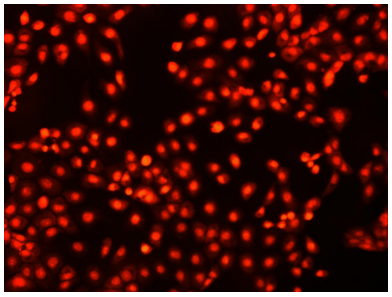


PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of gingerol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

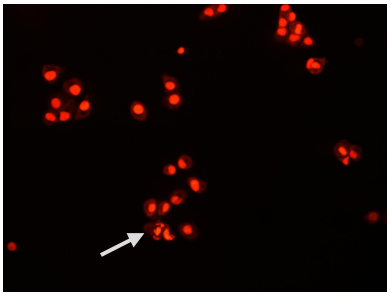
* $p<0.05$
** $p<0.01$

FIGURE 42 a

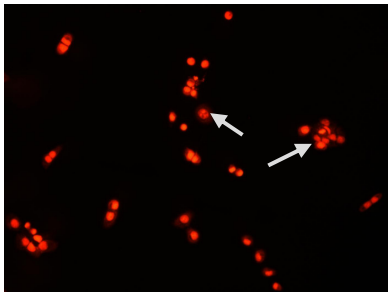
Effect of 6-gingerol on apoptosis in PC-3 cells



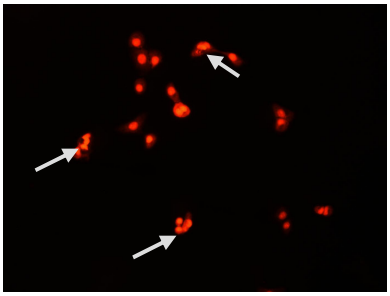
Control



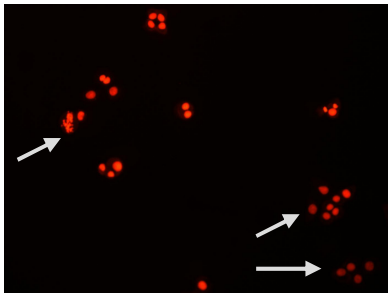
5 μM 6-gingerol



25 μM 6-gingerol



50 μM 6-gingerol

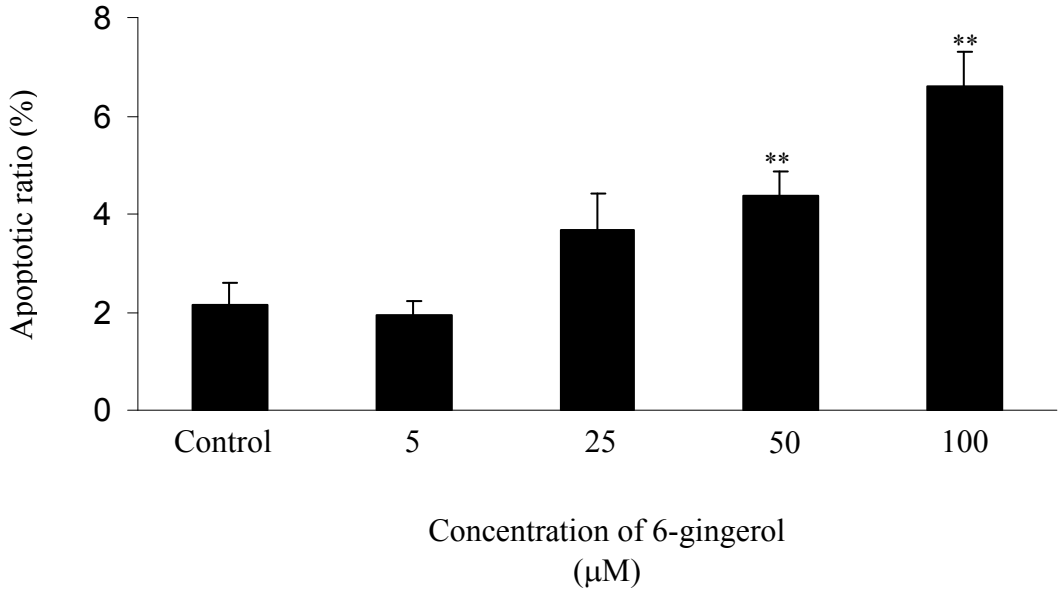


100 μM 6-gingerol

PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 42 b

Effect of 6-gingerol on apoptosis in PC-3 cells

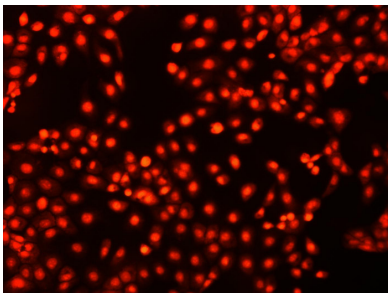


PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

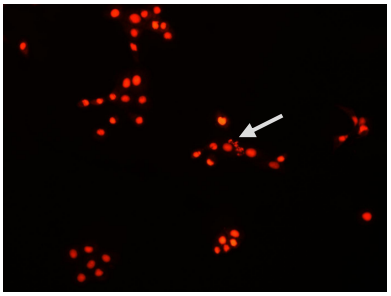
** $p<0.01$

FIGURE 43 a

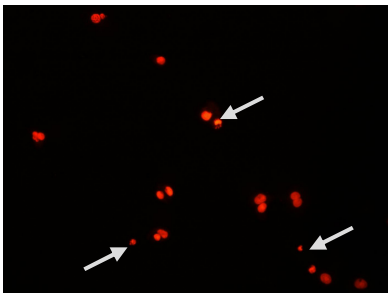
Effect of 8-gingerol on apoptosis in PC-3 cells



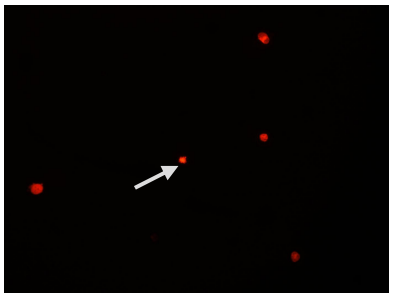
Control



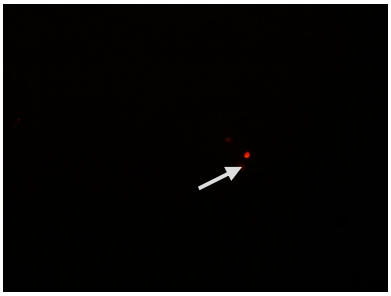
5 μM 8-gingerol



25 μM 8-gingerol



50 μM 8-gingerol

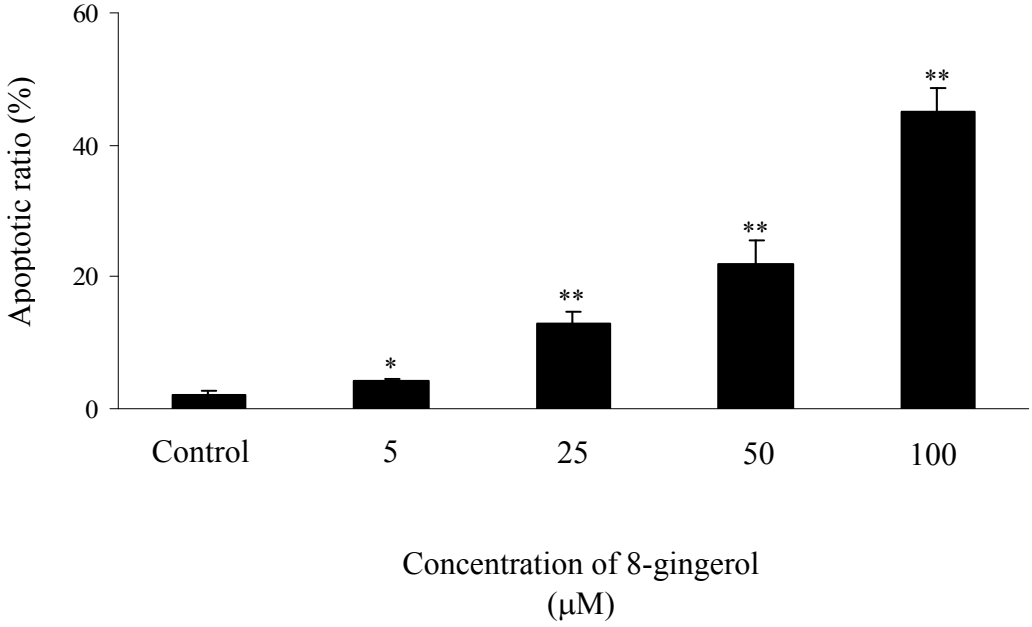


100 μM 8-gingerol

PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 43 b

Effect of 8-gingerol on apoptosis in PC-3 cells



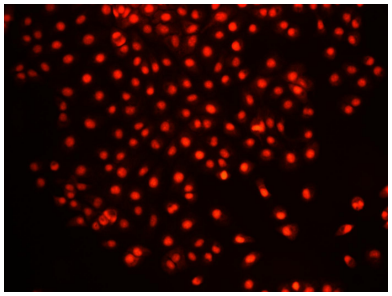
PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 8-gingerol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

* $p < 0.05$

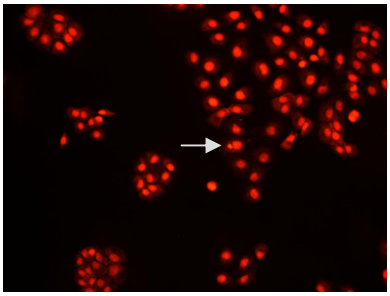
** $p < 0.01$

FIGURE 44 a

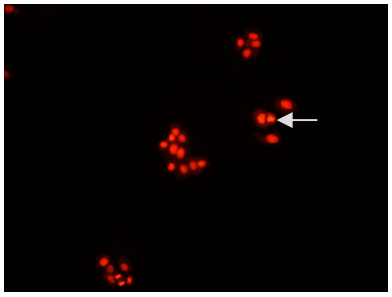
Effect of shogaol mixture on apoptosis in PC-3 cells



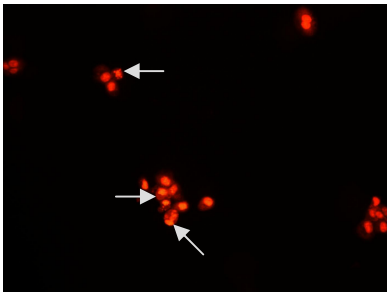
Control



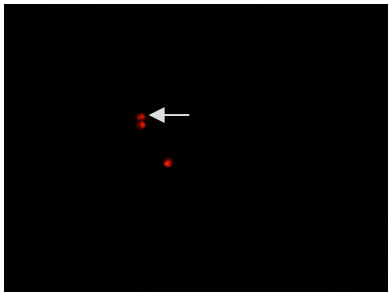
1.6 μg/mL shogaol mixture



8 μg/mL shogaol mixture



16 μg/mL shogaol mixture

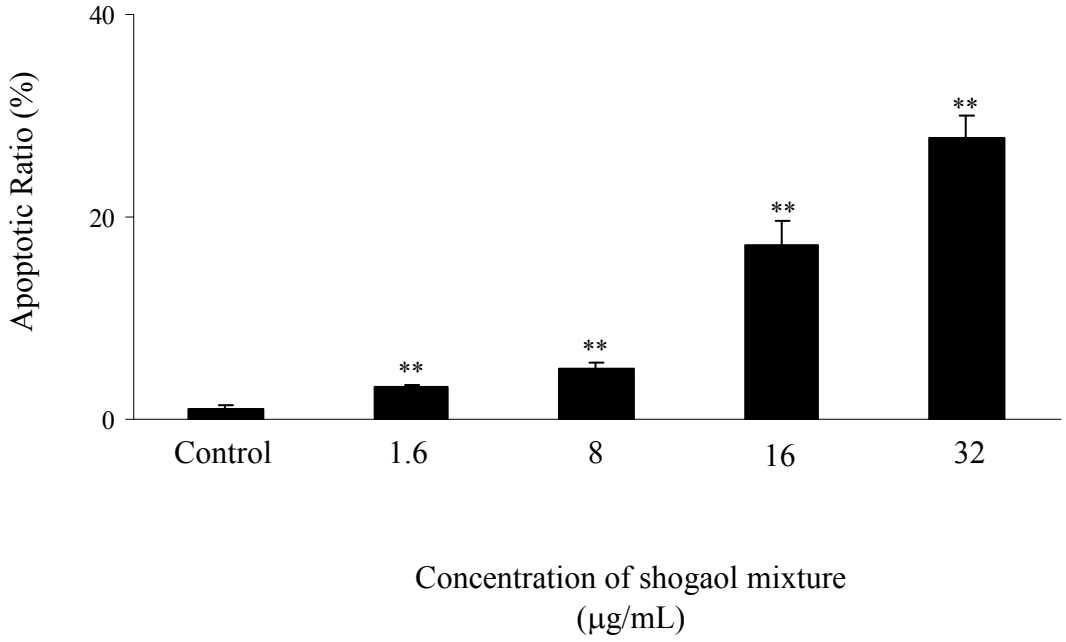


32 μg/mL shogaol mixture

PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 44 b

Effect of shogaol mixture on apoptosis in PC-3 cells

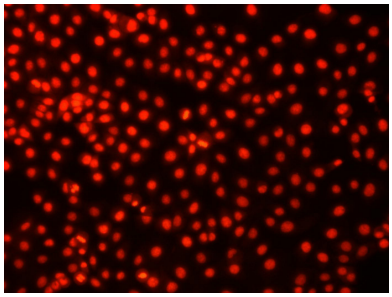


PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of shogaol mixture and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

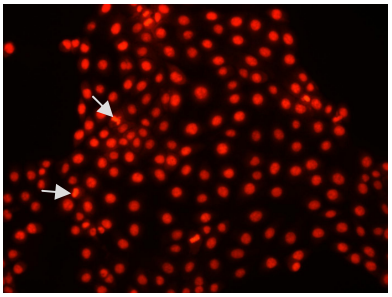
** $p<0.01$

FIGURE 45 a

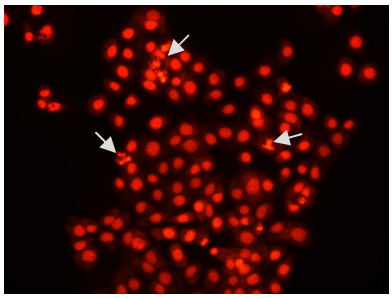
Effect of 6-shogaol on apoptosis in PC-3 cells



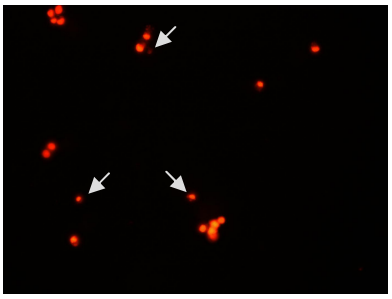
Control



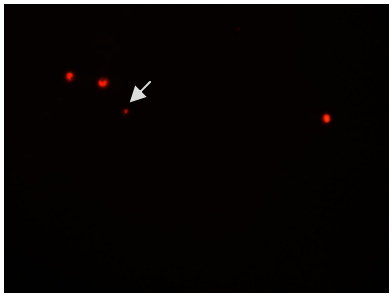
5 μM 6-shogaol



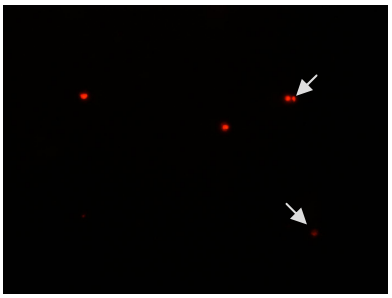
25 μM 6-shogaol



50 μM 6-shogaol



75 μM 6-shogaol

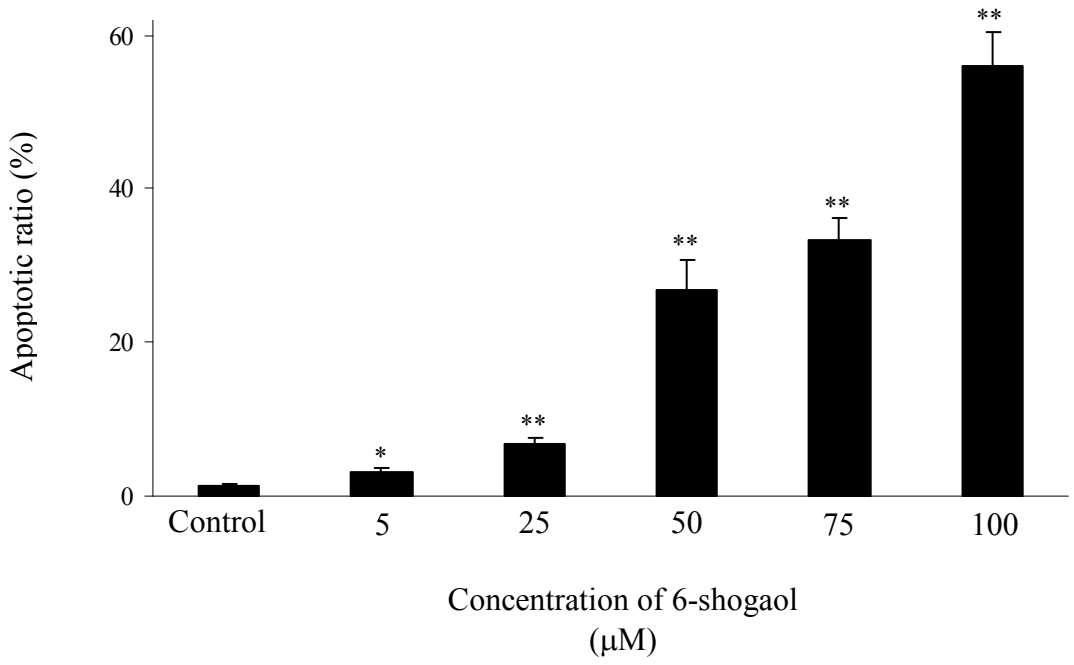


100 μM 6-shogaol

PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei as indicated by the white arrows.

FIGURE 45 b

Effect of 6-shogaol on apoptosis in PC-3 cells



PC-3 cells were seeded at a density of $1x10^4$ cells/mL and allowed to attach for 24 hours. The cells were then treated with different concentrations of 6-shogaol and incubated for 48 hours. The cells were stained with propidium iodide (1μg/mL) and viewed under the fluorescence microscope. Apoptotic cells were characterized by condensed and fragmented nuclei. [Apoptotic ratio = No. of apoptotic cells/total no. of cells]. p value was calculated using the Student *t* test, with respect to the control.

* $p<0.05$
** $p<0.01$

Effect of 6-gingerol, 8-gingerol and 6-shogaol on cell cycle measured using flow cytometry

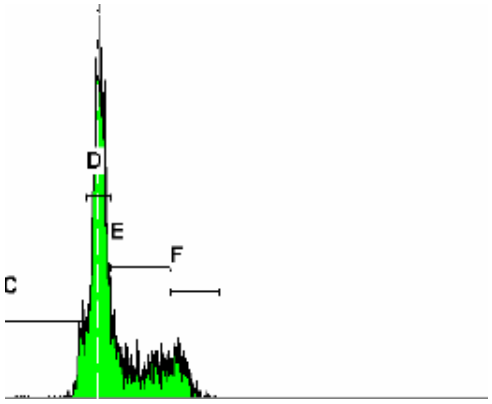
To further confirm the occurrence of apoptosis, we conducted cell cycle analysis as described previously. Cell cycle consists of 4 basic phases, the G1 (gap), S (synthesis), G2 (gap) and M (mitosis) phase. During these phases, different amount of DNA is present in these cells that can be measured using flow cytometry. In the G1 phase of cell cycle (resting phase), the cells contain diploid amount of DNA (2N). When entering into the S phase, DNA multiplication takes place until it doubles in number (between 2N-4N). From here when cells enter the G2M phase they have 4N number of DNA. This can be measured by using a dye that can bind with the DNA, such as propidium iodide (PI). However PI can bind to both DNA and RNA; for this reason we use RNAse A to destroy the RNA thus enabling PI to only bind to DNA in the cell. By measuring the fluorescence of the cell by flow cytometry, we can determine which phase each cell is in [316]. In short, LNCaP and PC-3 cells were treated with different concentrations of 6-gingerol, 8-gingerol and 6-shogaol and incubated for 48 hours. At the end of the treatment, the cells were harvested, lysed with ethanol and stained with PI in the presence of RNAse A. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells in each phase of the cell cycle was calculated using cytologic software from Coulter Corp. Based on the extent of staining in each cell the percentage of cells in each phase of the cell cycle can be analyzed. The cells in the G1 phase will contain 2N number of DNA and cells in the M phase 4N number of DNA (after replication). The S phase which lies between these two phases, the cells will contain between 2N-4N numbers of DNA. When the cells are arrested before entering

the G1 phase, apoptosis is said to have occurred and this is often referred to as sub G1 phase.

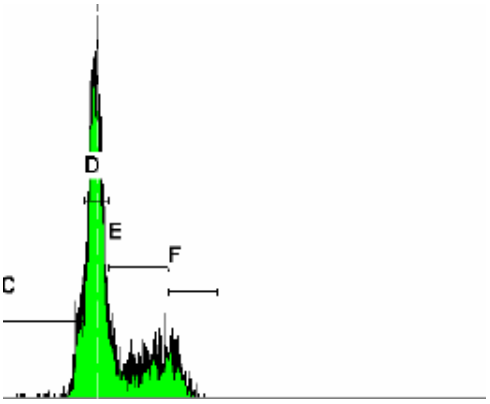
6-gingerol was able to arrest cells in the subG1 or apoptotic phase at the highest concentration tested in LNCaP cells only. However in PC-3 cells, 6-gingerol was not able to arrest cells in the subG1 phase but was able to significantly arrest cells in the G2M phase of the cell cycle. 8-gingerol on the other hand was able to induce apoptosis in LNCaP cells at both 75 μM and 100 μM as seen with sub G1 arrest but not any other phase of the cell cycle. However in PC-3 cells, it was able to induce arrest in both the sub G1 phase (at all concentrations tested) and the synthesis (S) phase of the cell cycle (at 50, 75 and 100 μM). 6-shogaol was able to induce apoptosis as seen with sub G1 phase arrest in both cell lines. 6-shogaol was also able to induce S phase arrest in LNCaP cells (at 50 and 75 μM) and PC-3 cells (at 50, 75 and 100 μM). The results are shown in Figures 46-51.

FIGURE 46 a

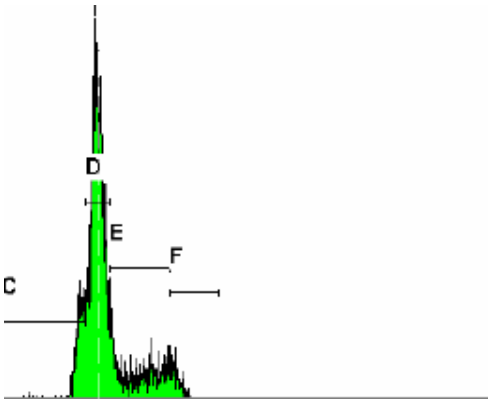
Effect of 6-gingerol on cell cycle in LNCaP cells



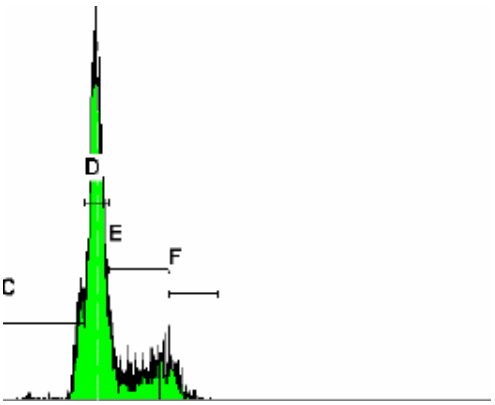
Control



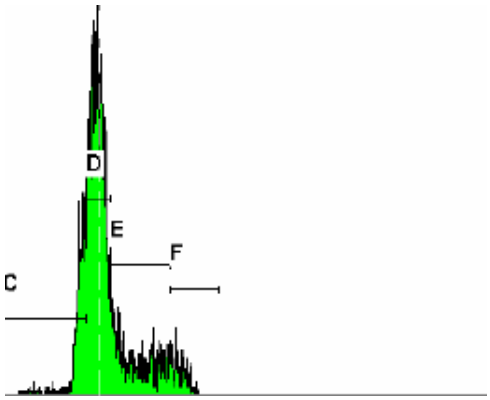
5 µM 6-gingerol



25 µM 6-gingerol



50 µM 6-gingerol

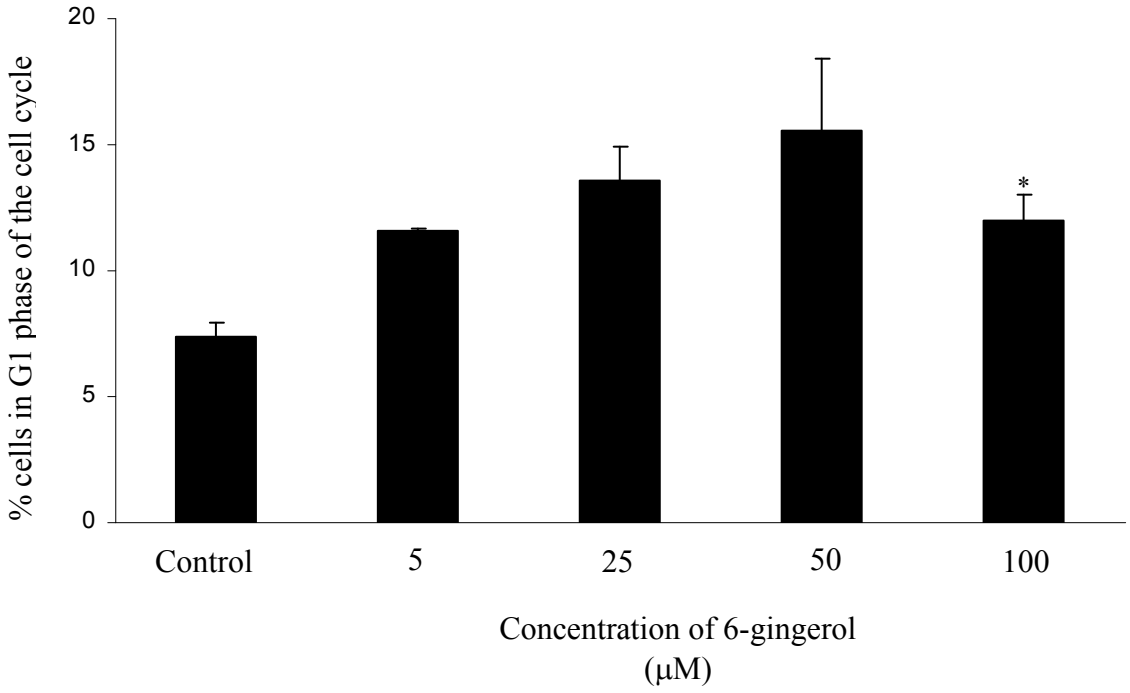


100 µM 6-gingerol

LNCaP cells were treated with different concentrations of 6-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 µg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry.

FIGURE 46 b

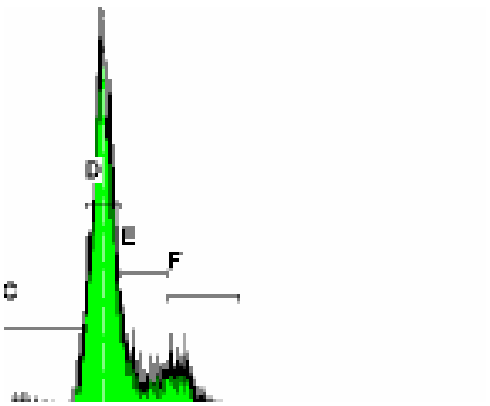
Effect of 6-gingerol on cell cycle in LNCaP cells



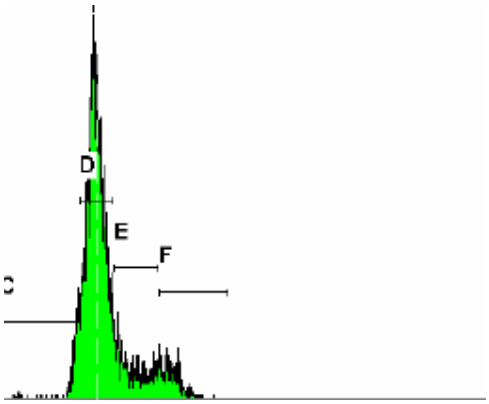
LNCaP cells were treated with different concentrations of 6-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.
* $p<0.05$

FIGURE 47 a

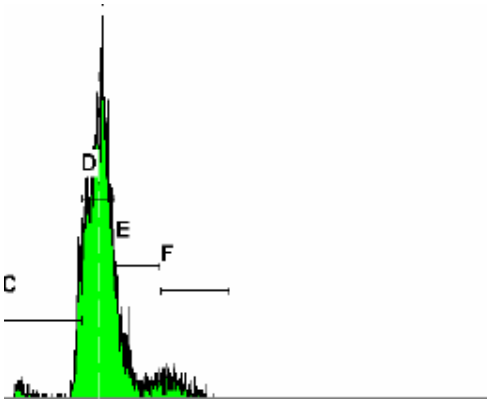
Effect of 8-gingerol on cell cycle in LNCaP cells



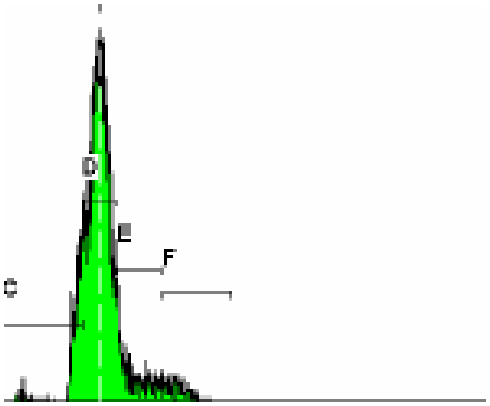
Control



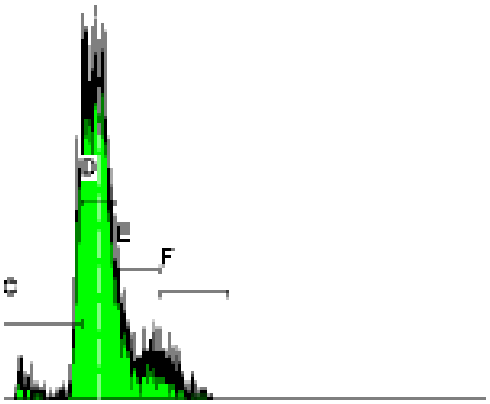
5 µM 8-gingerol



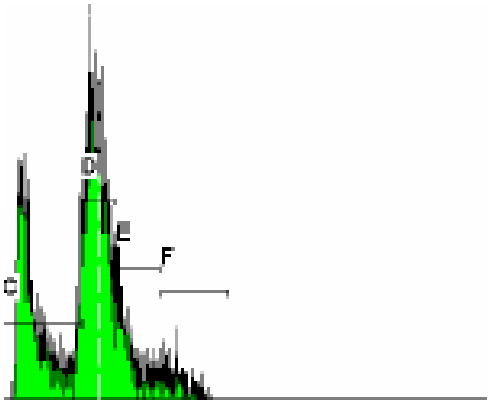
25 µM 8-gingerol



50 µM 8-gingerol



75 µM 8-gingerol

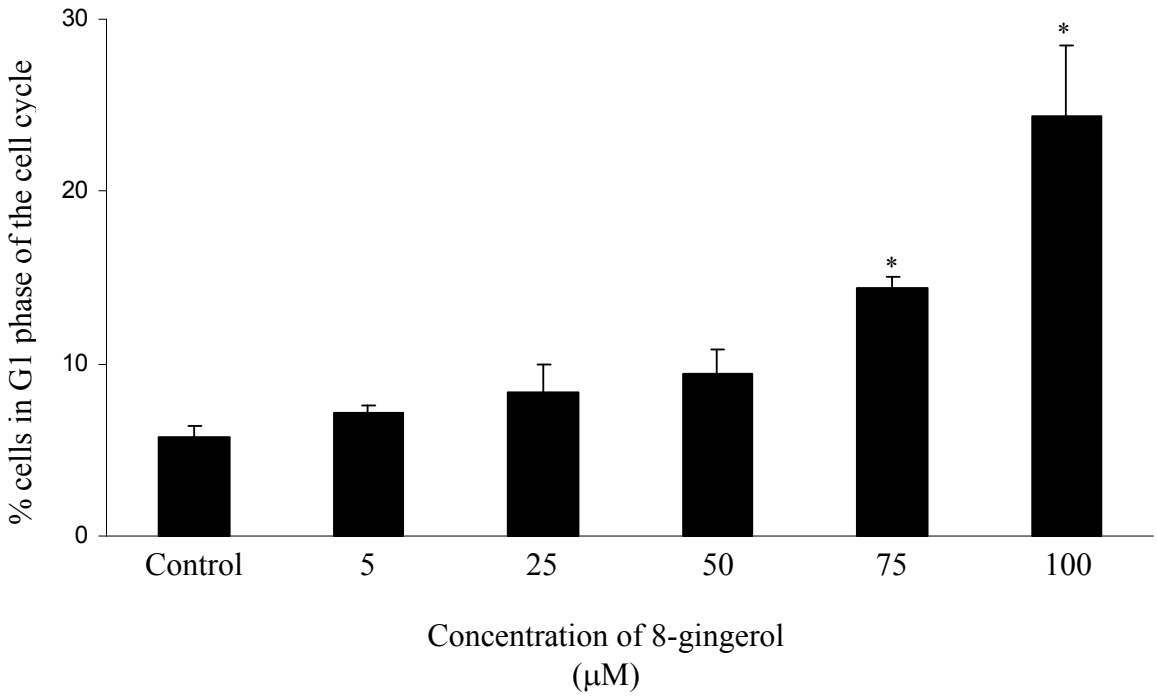


100 µM 8-gingerol

LNCaP cells were treated with different concentrations of 8-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 µg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry.

FIGURE 47 b

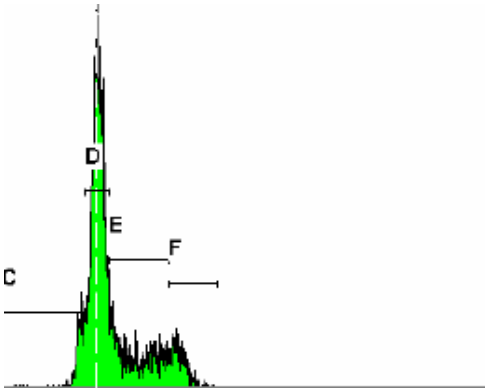
Effect of 8-gingerol on cell cycle in LNCaP cells



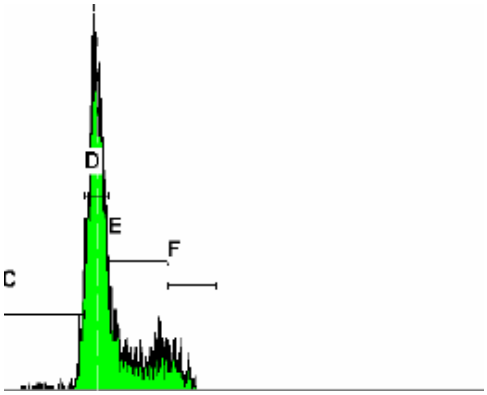
LNCaP cells were treated with different concentrations of 8-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.
* $p<0.05$

FIGURE 48 a

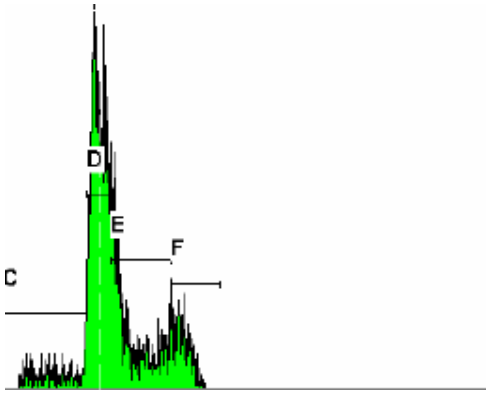
Effect of 6-shogaol on cell cycle in LNCaP cells



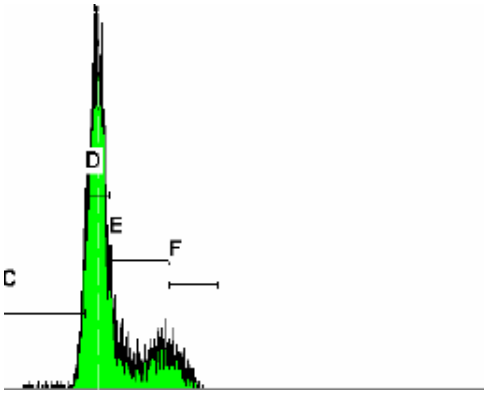
Control



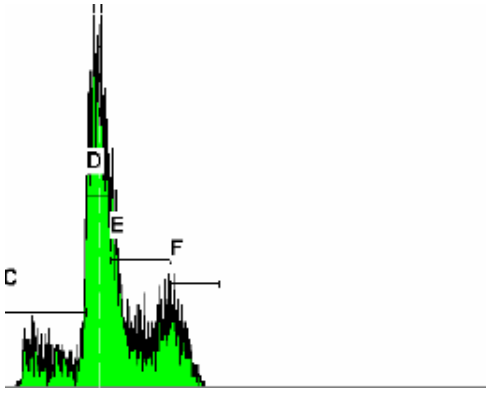
5 μM 6-shogaol



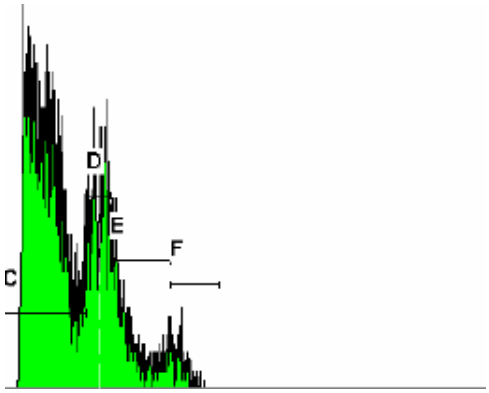
25 μM 6-shogaol



50 μM 6-shogaol



75 μM 6-shogaol

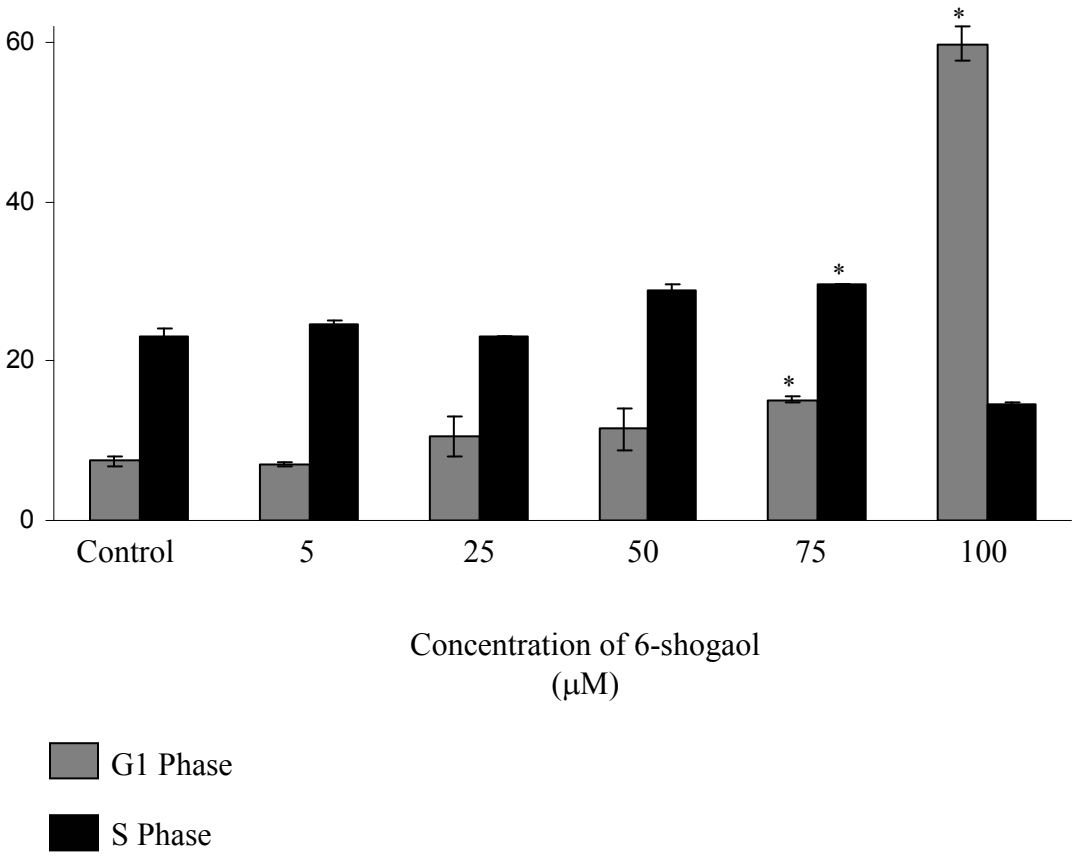


100 μM 6-shogaol

LNCaP cells were treated with different concentrations of 6-shogaol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry.

FIGURE 48 b

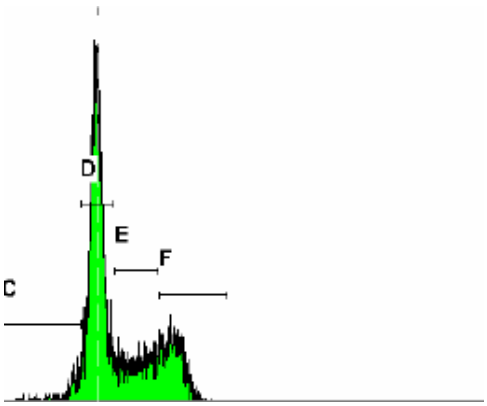
Effect of 6-shogaol on cell cycle in LNCaP cells



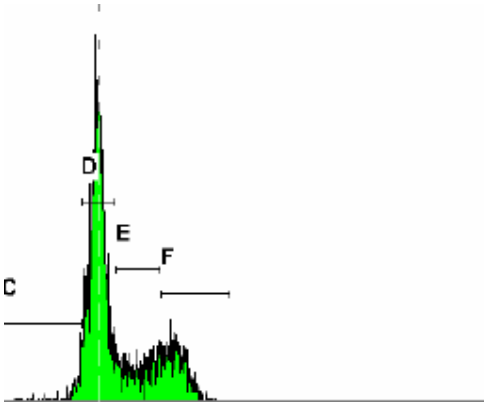
LNCaP cells were treated with different concentrations of 6-shogaol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.
* $p<0.05$

FIGURE 49 a

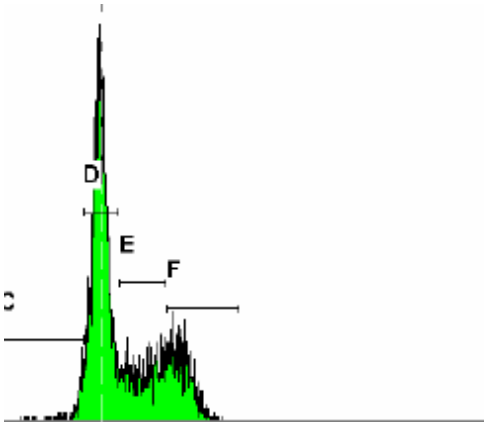
Effect of 6-gingerol on cell cycle in PC-3 cells



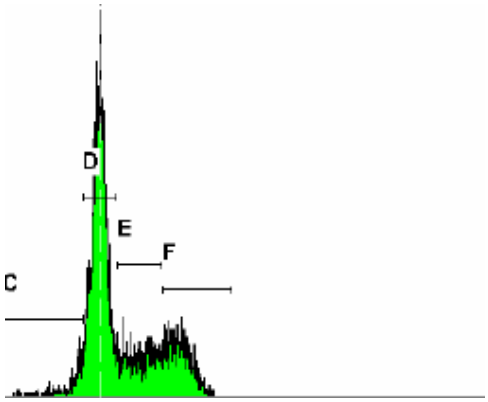
Control



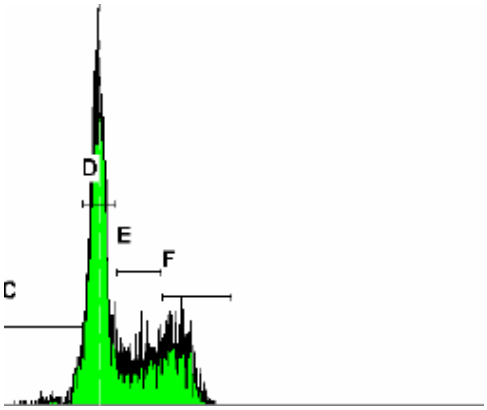
5 µM 6-gingerol



25 µM 6-gingerol



50 µM 6-gingerol

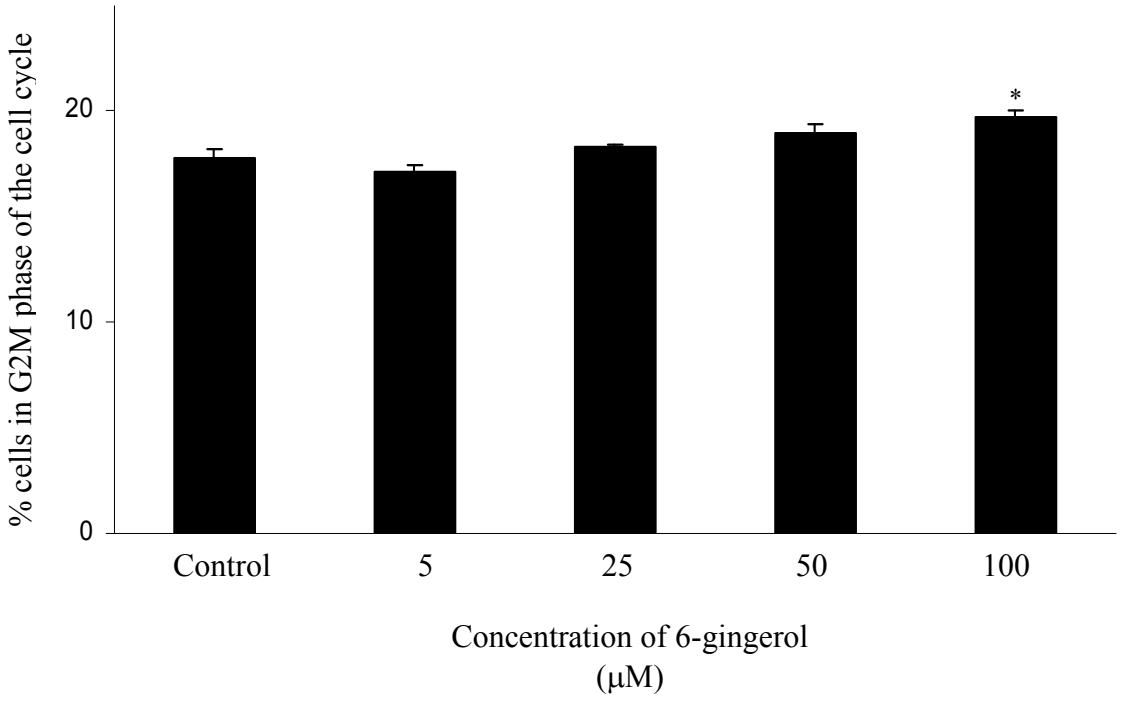


100 µM 6-gingerol

PC-3 cells were treated with different concentrations of 6-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 µg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry.

FIGURE 49 b

Effect of 6-gingerol on cell cycle in PC-3 cells

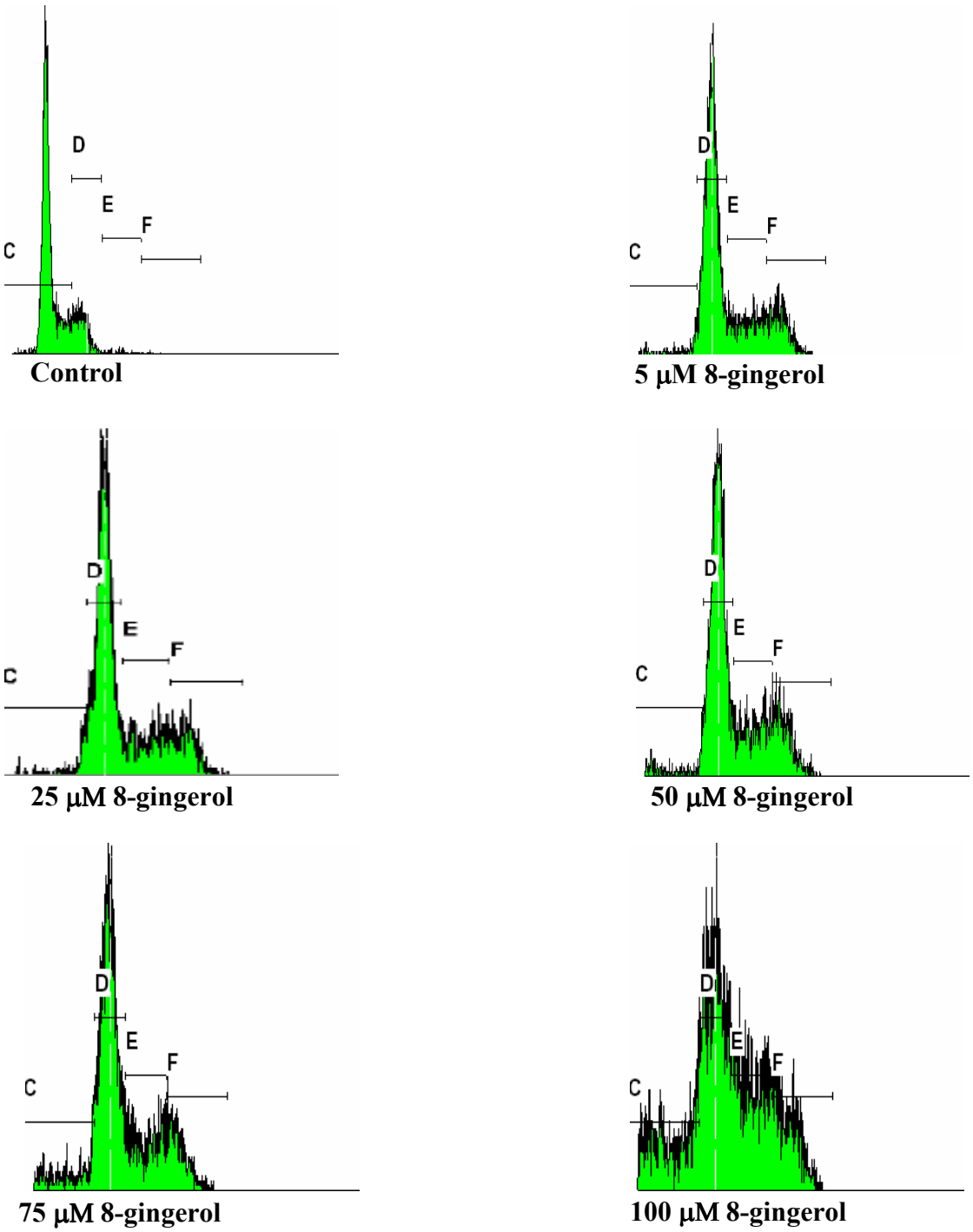


PC-3 cells were treated with different concentrations of 6-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.

* $p<0.05$

FIGURE 50 a

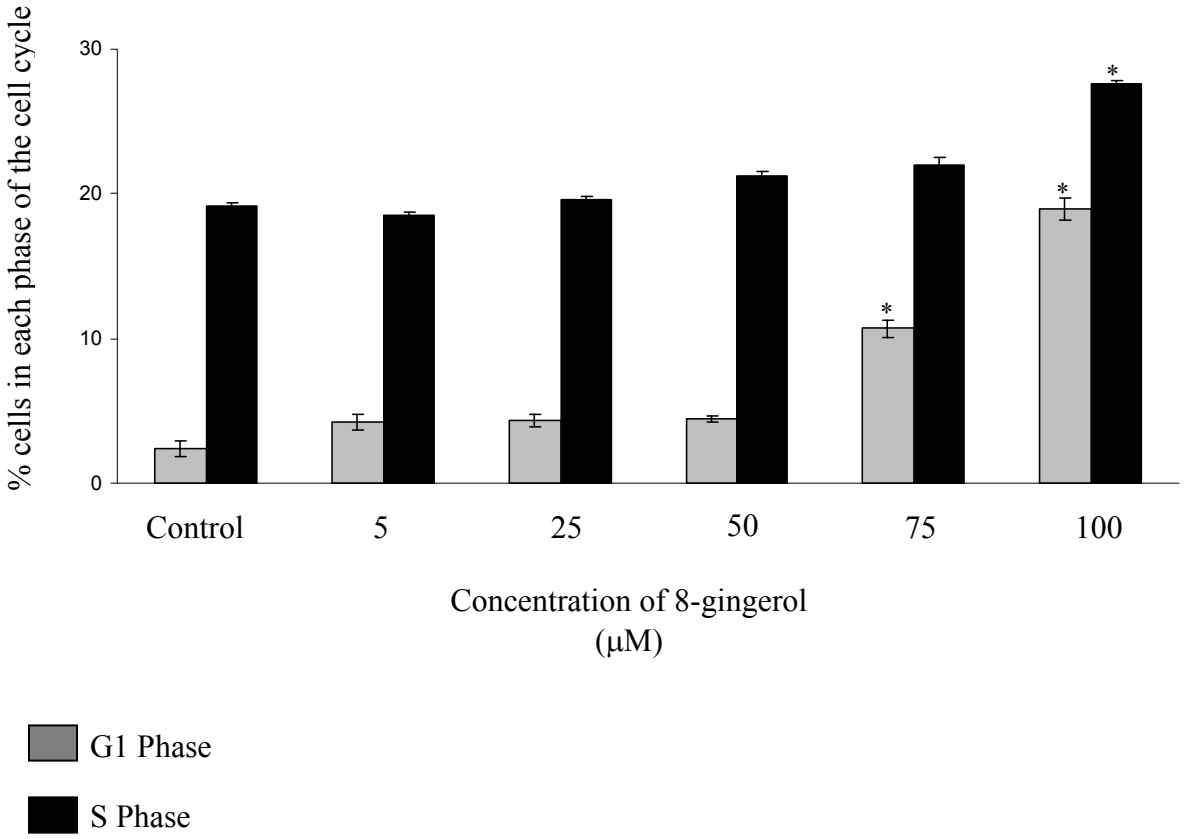
Effect of 8-gingerol on cell cycle in PC-3 cells



PC-3 cells were treated with different concentrations of 8-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 µg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry.

FIGURE 50 b

Effect of 8-gingerol on cell cycle in PC-3 cells

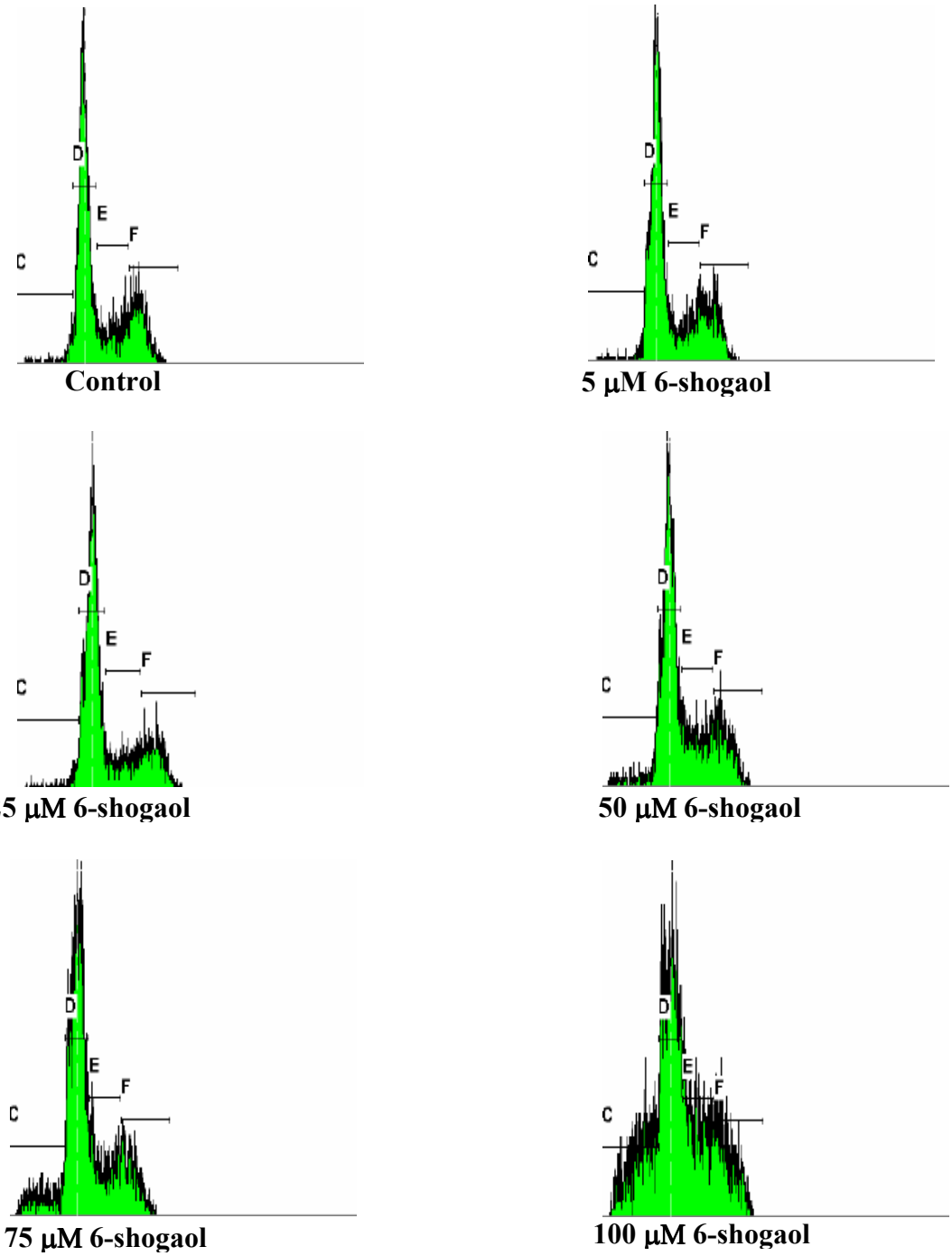


PC-3 cells were treated with different concentrations of 8-gingerol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.

* $p<0.05$

FIGURE 51 a

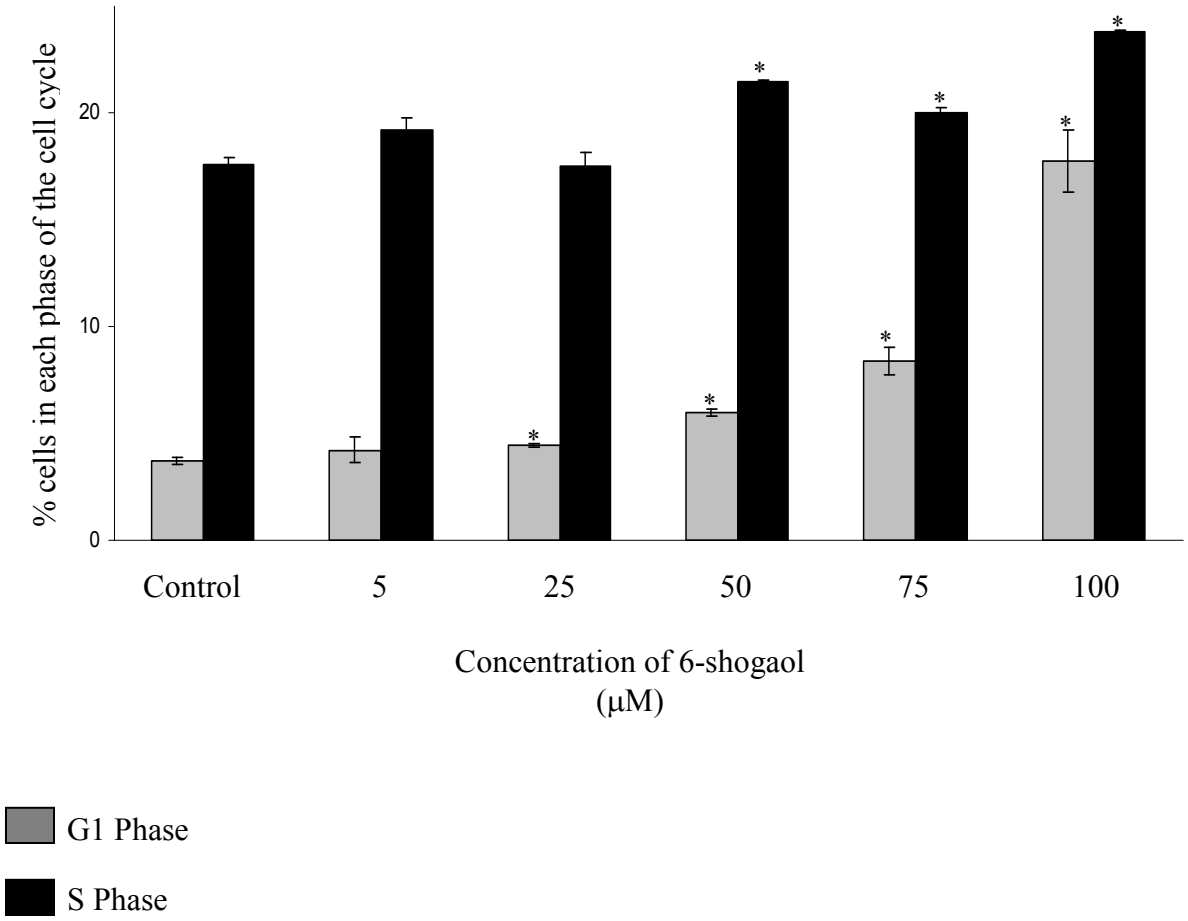
Effect of 6-shogaol on cell cycle in PC-3 cells



PC-3 cells were treated with different concentrations of 6-shogaol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 µg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry

FIGURE 51 b

Effect of 6-shogaol on cell cycle in PC-3 cells



PC-3 cells were treated with different concentrations of 6-shogaol for 48 hours. The cells were washed, lysed with ice cold ethanol and placed on ice. The cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 10 μg/mL propidium iodide. The cells were then incubated for 30 minutes and analyzed by flow cytometry. The values are mean + standard error of percentage of cells in the subG1 phase of the cell cycle. p value was calculated using the Student *t* test, with respect to the control.
* $p<0.05$

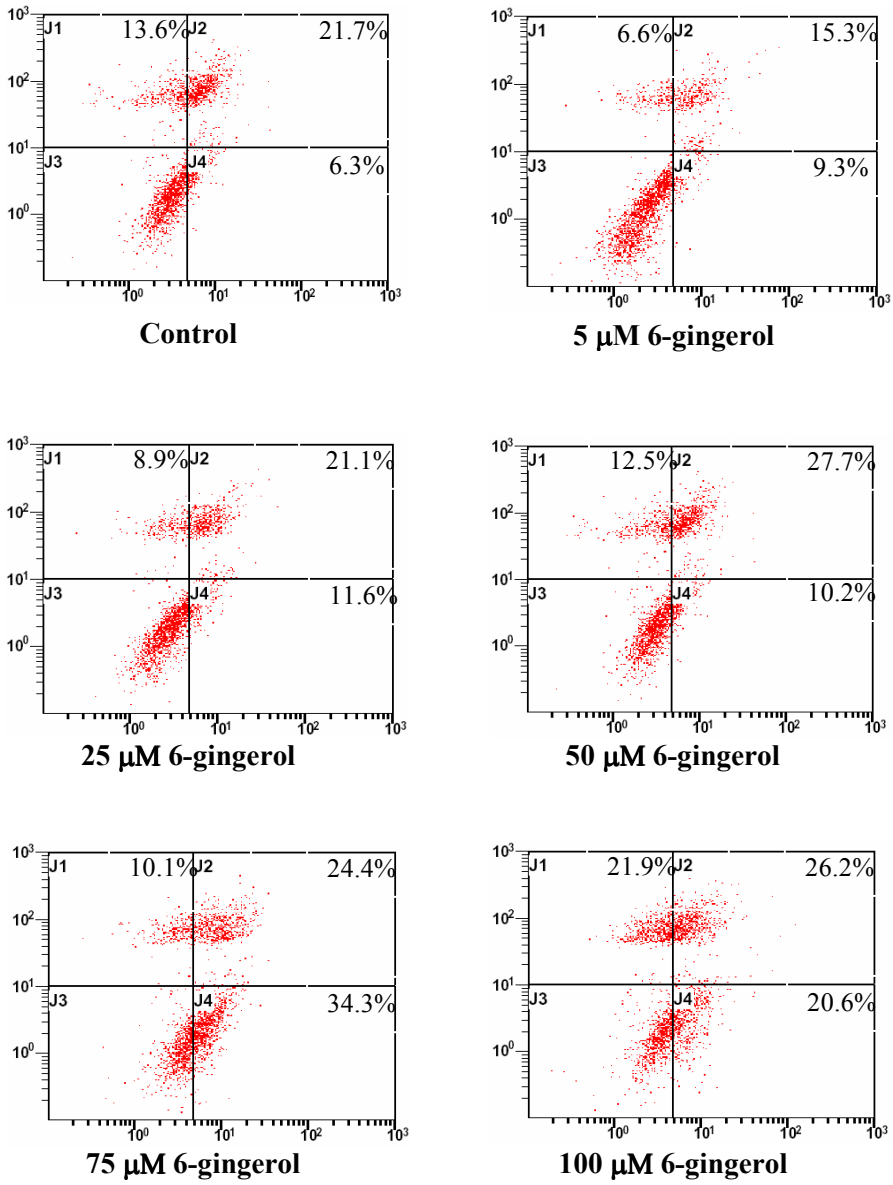
Effect of 6-gingerol, 8-gingerol and 6-shogaol on apoptosis measured using Annexin V-FITC and Propidium iodide (PI) co-staining

The presence of apoptosis was further confirmed using Annexin V-FITC and PI staining. During the process of apoptosis, one of the early events that takes place is the externalization of phosphotidyl serine (PS), a phospholipids normally located in the inner portion of the cell membrane. This can be measured using Annexin V, a protein that binds specifically to PS. PI on the other hand can enter and bind to nucleic acid only in cells whose membrane has been ruptured. Hence by measuring the levels of both these dyes, we can estimate the number of viable, apoptotic and necrotic cells in the sample of interest based on the flow cytometric data. Cells in the lower left hand quadrant indicate viable cells (that are both PI and Annexin V negative). The cells in the lower right hand quadrant are cells which are only Annexin V positive which indicates the percentage of apoptotic cells. Cells in the upper right hand quadrant are both PI and Annexin V positive and are said to be late apoptotic. Cells in the upper left hand quadrant are only PI positive and are considered necrotic. Cells undergoing apoptosis are present either in the lower or upper right hand quadrant. For performing this assay LNCaP and PC-3 cells were treated with the respective compounds and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V –PI reagent for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

Based on the flow cytometric analysis, 6-gingerol did not induce apoptosis in either LNCaP (Figure 52) or PC-3 (Figure 55) cells. Most of the cells in the control as well as the treatment group were present in the lower left hand quadrant. 8-gingerol and 6-shogaol induced apoptosis in both LNCaP and PC-3 cells. Both these compounds were able to induce apoptosis in LNCaP cells (as seen in the lower right hand quadrant) at 50, 75 and 100 μM (Figures 53-54). However in PC-3 cells, both 6-shogaol and 8-gingerol could induce apoptosis only at higher concentrations and the cells seemed to be late apoptotic (upper right quadrant). 8-gingerol was able to induce apoptosis only at 100 μM (Figure 56) but 6-shogaol induced apoptosis at 75 and 100 μM (Figure 57). This indicated that the time course and probably the mechanisms of action might be different in LNCaP and PC-3 cells by 8-gingerol and 6-shogaol.

FIGURE 52

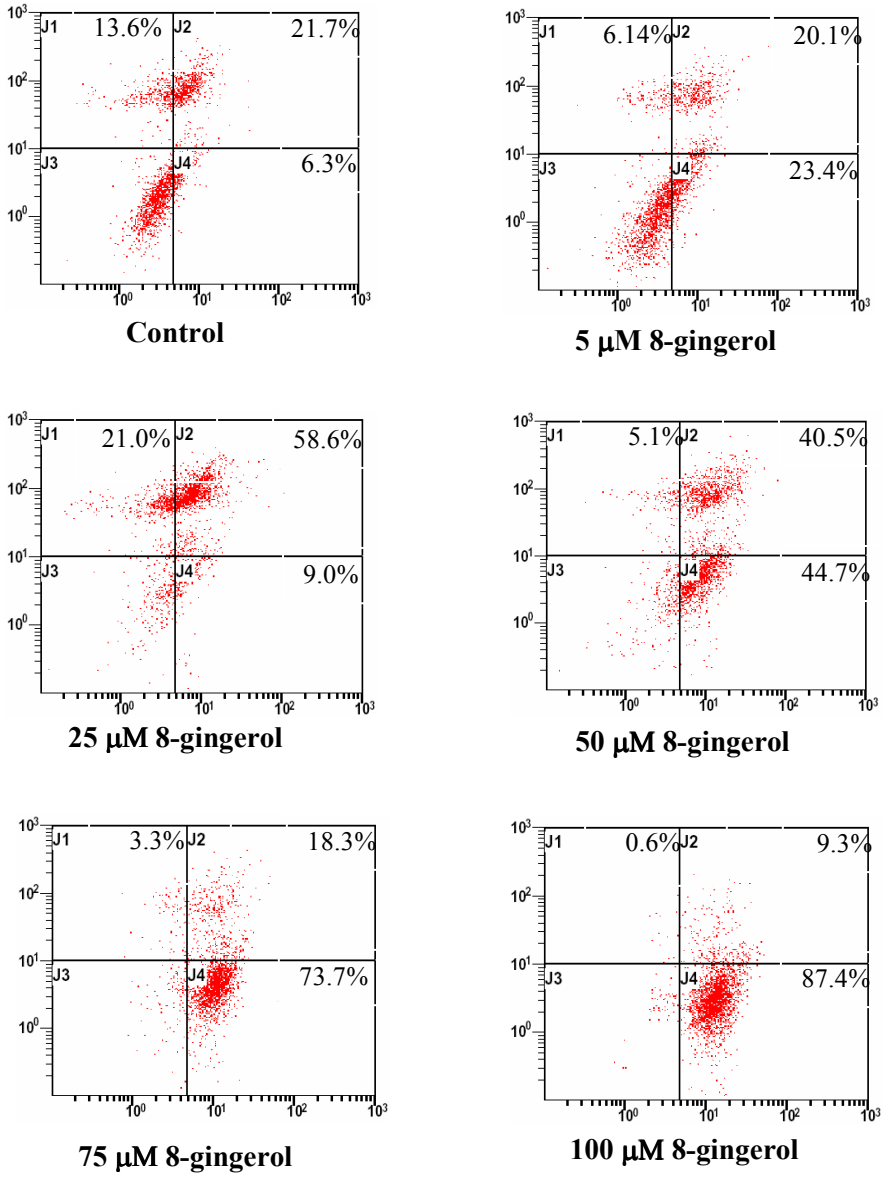
Effect of 6-gingerol on apoptosis in LNCaP cells measured using Annexin V-FITC and PI staining



LNCaP cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 6-gingerol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

FIGURE 53

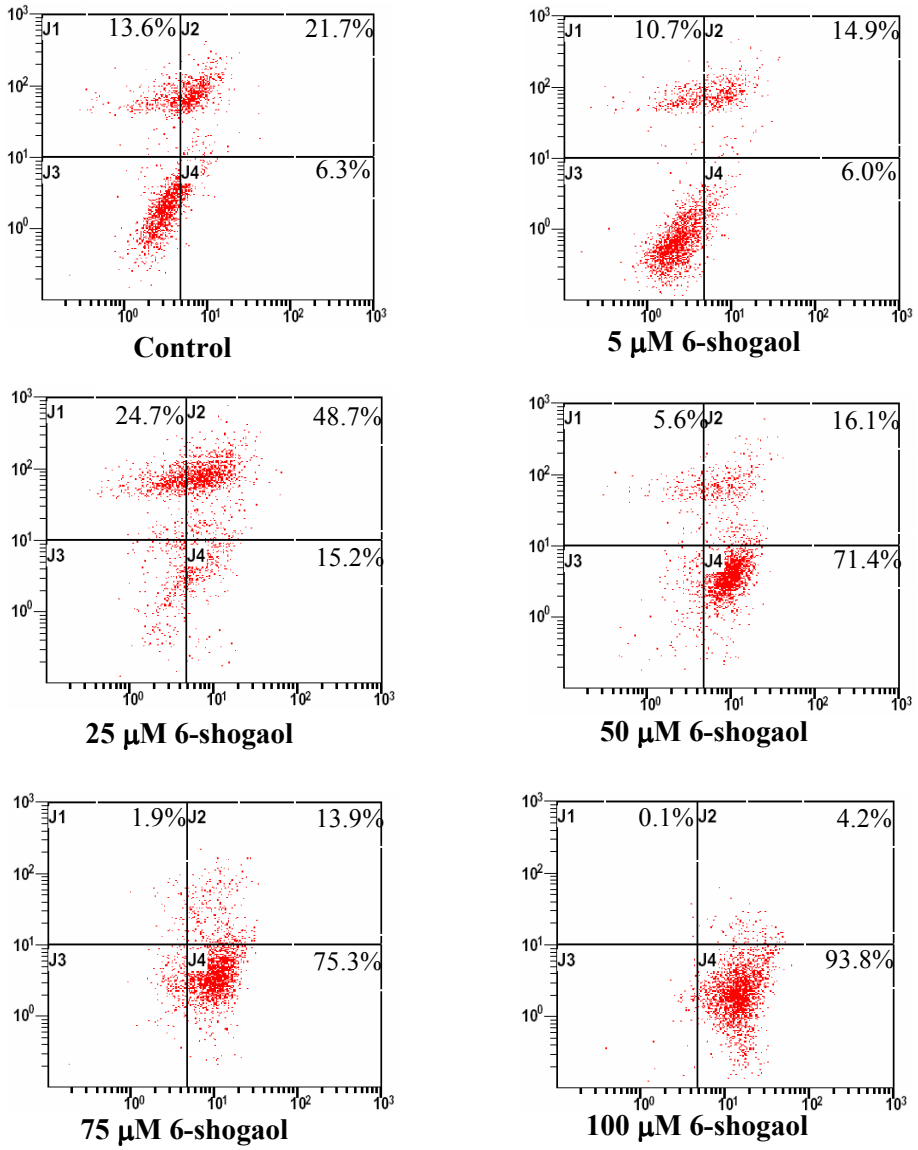
Effect of 8-gingerol on apoptosis in LNCaP cells measured using Annexin V-FITC and PI staining



LNCaP cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 8-gingerol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

FIGURE 54

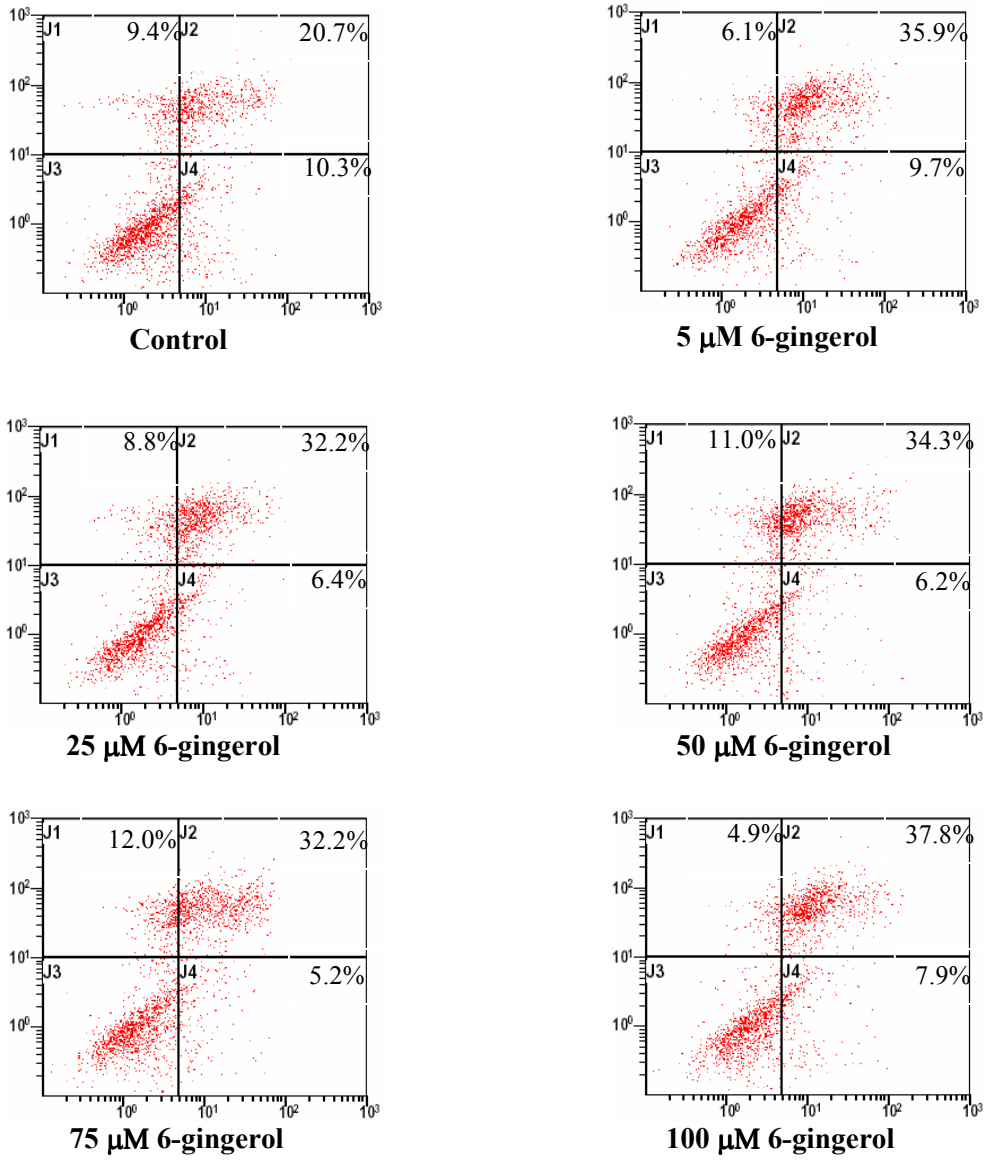
Effect of 6-shogaol on apoptosis in LNCaP cells measured using Annexin V-FITC and PI staining



LNCaP cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 6-shogaol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

FIGURE 55

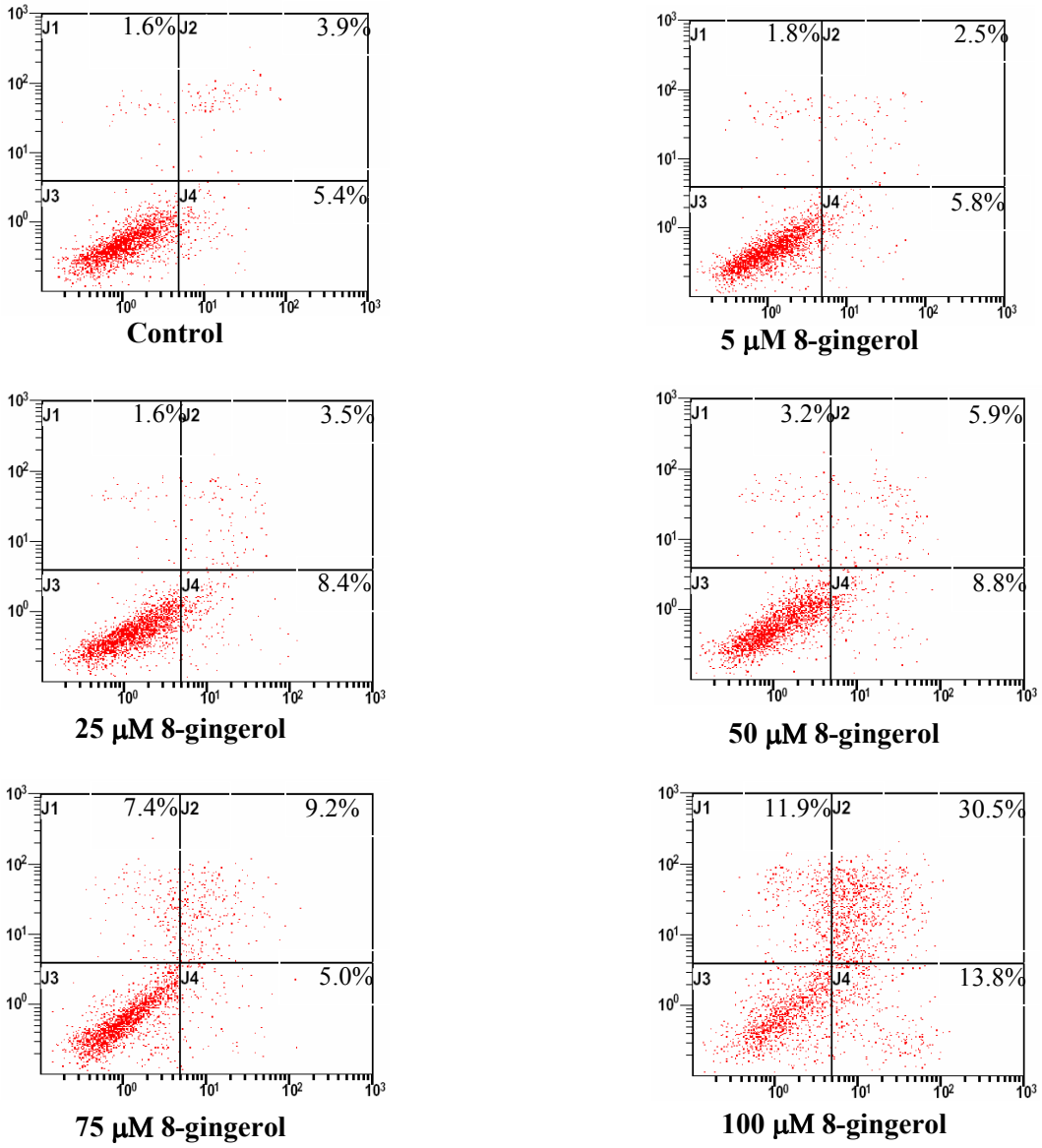
Effect of 6-gingerol on apoptosis in PC-3 cells measured using Annexin V-FITC and PI staining



PC-3 cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 6-gingerol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

FIGURE 56

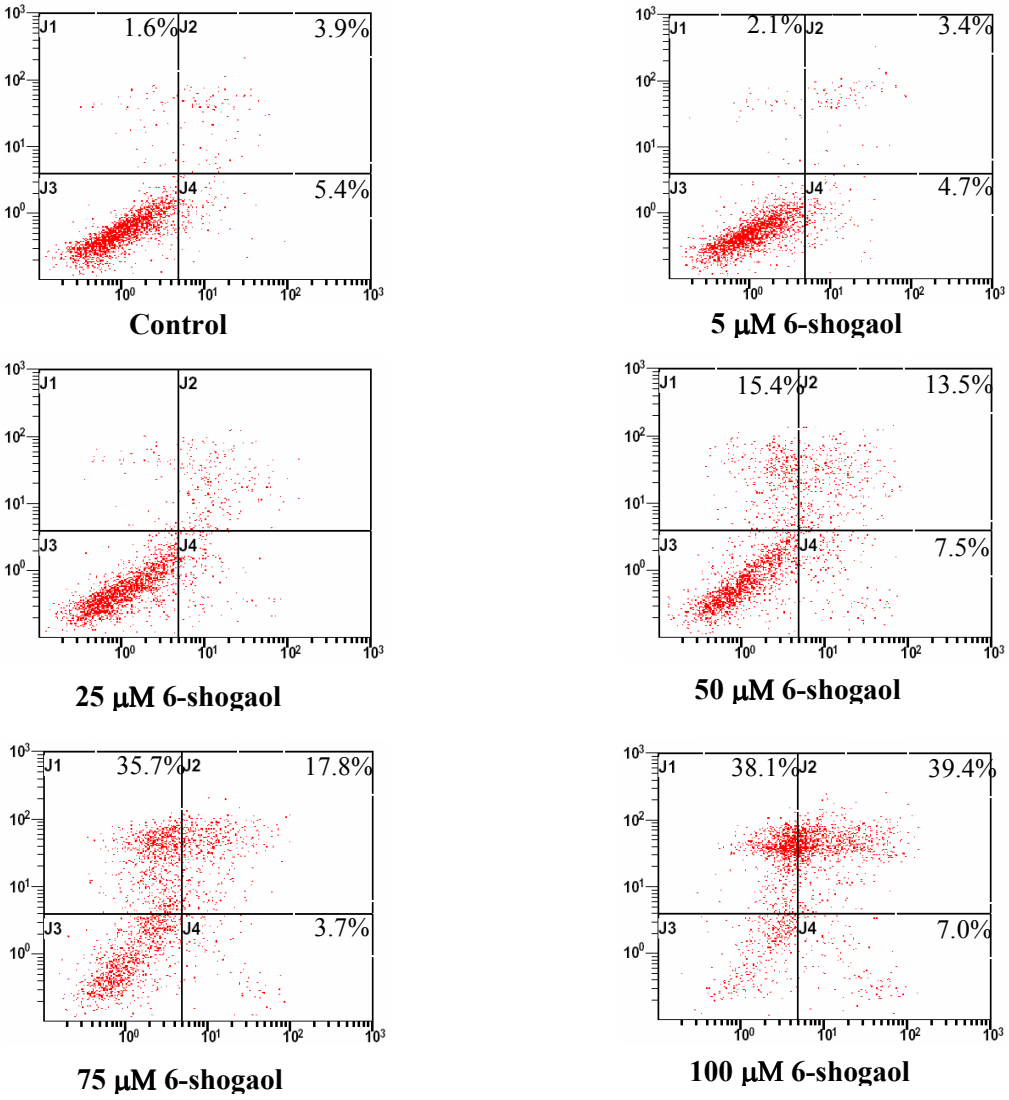
Effect of 8-gingerol on apoptosis in PC-3 cells measured using Annexin V-FITC and PI staining



PC-3 cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 8-gingerol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

FIGURE 57

Effect of 6-shogaol on apoptosis in PC-3 cells measured using Annexin V-FITC and PI staining



PC-3 cells were plated at a concentration of $2x10^5$ cells/well in 6-well culture dishes and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. The cells were then treated with different concentrations of 6-shogaol and incubated for an additional 48 hours. At the end of the treatment, the cells were harvested, washed with PBS twice and incubated with Annexin V-FITC (0.25 µg/mL) and PI (5 µg/mL) for 15 minutes at room temperature. The cells were then analyzed using a Coulter Epics-Profile II flow cytometer and the proportion of cells stained with Annexin V, PI or both were calculated by using cytologic software from Coulter Corp.

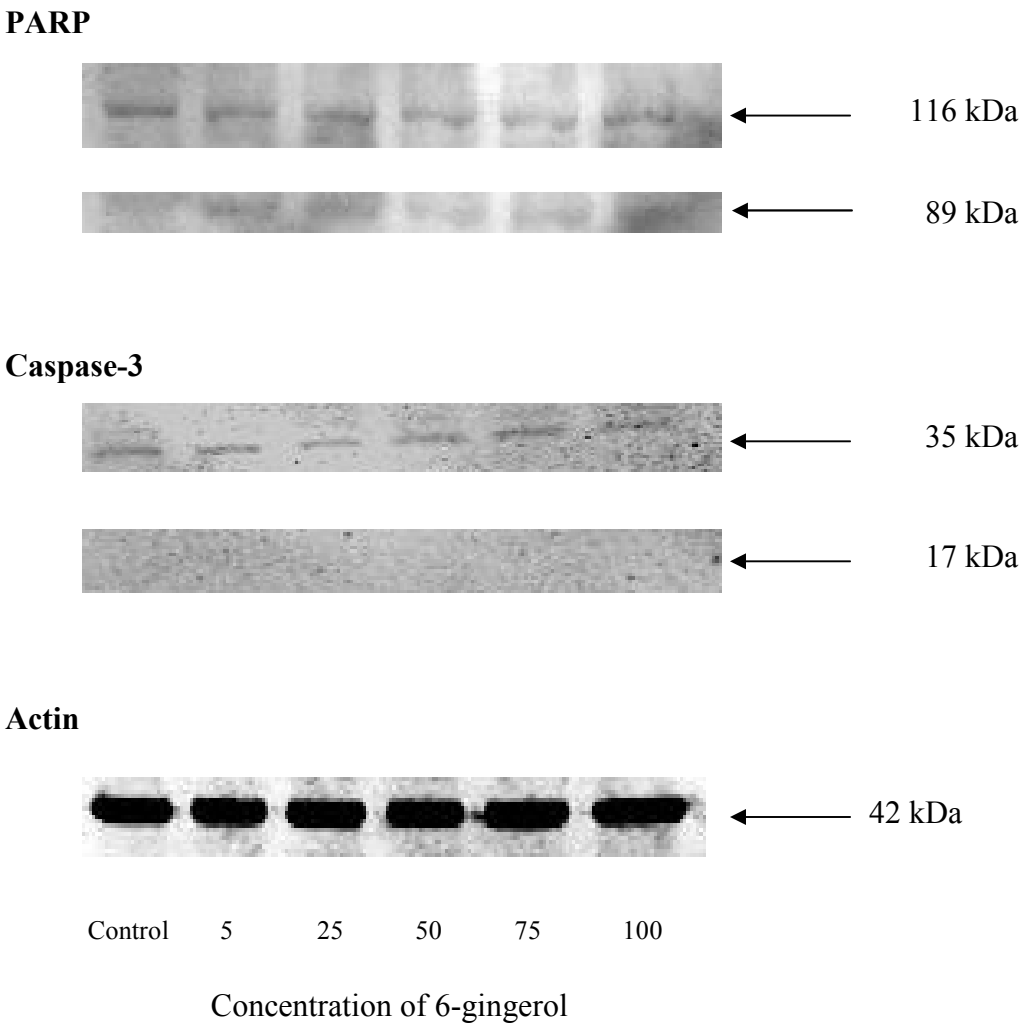
Effect of 6-gingerol, 8-gingerol and 6-shogaol on apoptosis measured using Western blot analysis

To further confirm the presence of apoptosis and to determine the mechanisms of action of the test compounds western blot analysis was performed. LNCaP or PC-3 cells were plated on 100 mm^2 Petri dishes and treated with different concentrations of the test compounds. Following incubation for 48 hours, proteins from the samples were extracted based on established protocols using RIPA buffer as described previously. The protein content in the samples was determined using a protein assay kit. The samples were then loaded onto a freshly prepared 8-14% polyacrylamide gel, electrophoresed at 70-120 V for 2-3 hours and the proteins were transferred onto nitrocellulose membranes at 100 V for 2 hours. The nitrocellulose membrane was blocked and probed with primary antibody for 12-18 hours at 4 °C with constant shaking. The membrane was then incubated with secondary antibody at room temperature for one hour and visualized to detect the presence of proteins. To detect the presence of apoptosis, we used PARP as the marker. PARP is a protein that is present downstream of the caspase cascade of apoptosis cleavage and is commonly used as a marker of apoptosis. During apoptosis this 116 kDa protein is cleaved into its 89 kDa cleavage product. To determine if the compounds were acting through the intrinsic or extrinsic pathway of apoptosis we used caspase-9 and caspase-8 antibodies respectively. The presence of apoptosis was seen as a cleavage product in the western blots.

As noted with the previous experiments, 6-gingerol did not induce apoptosis in LNCaP and PC-3 cells (Figure 58 and 59). No cleavage of both PARP or caspase-3

FIGURE 58

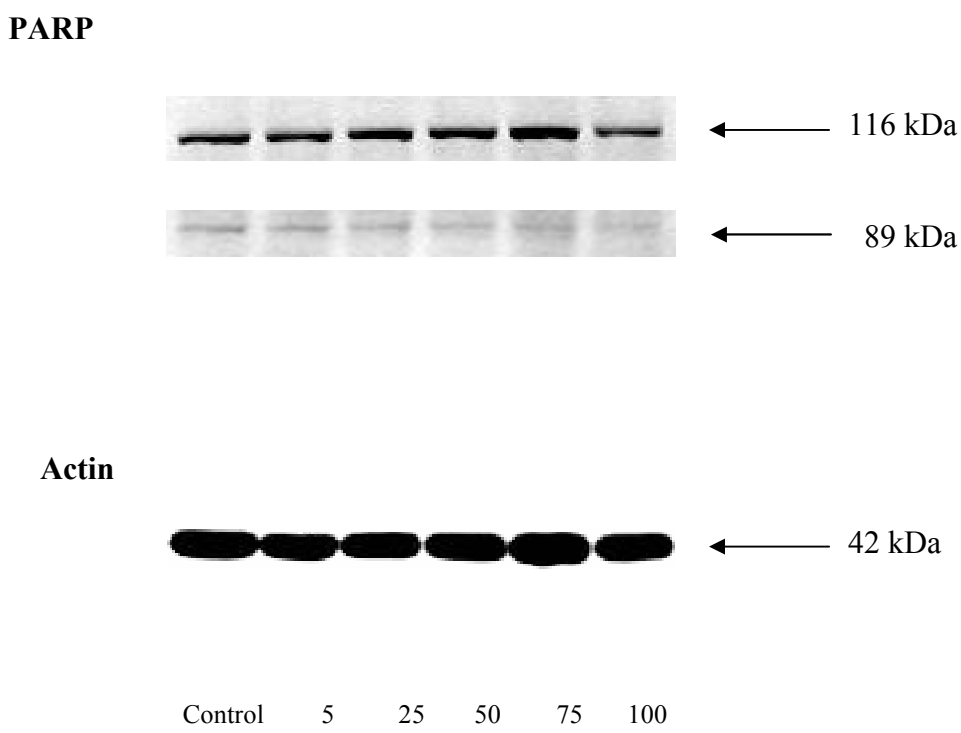
Effect of 6-gingerol on expression of apoptotic proteins in LNCaP cells



LNCaP cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 6-gingerol for 48 hours. At the end of the treatment, protein from the cells were extracted using RIPA buffer containing 1% protease inhibitor and phosphatase inhibitor cocktails, according to established protocols as described in materials and methods. The proteins in samples were determined using a BCA protein assay kit. Equal amounts (30 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The electrophoresed were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band (shown in the above figures) as described earlier.

FIGURE 59

Effect of 6-gingerol on expression of apoptotic proteins in PC-3 cells



PC-3 cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 6-gingerol for 48 hours. At the end of the treatment, protein from the cells were extracted using RIPA buffer containing 1% protease inhibitor and phosphatase inhibitor cocktails, according to established protocols as described in materials and methods. The proteins in samples were determined using a BCA protein assay kit. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The electrophoresed were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band (shown in the above figures) as described earlier

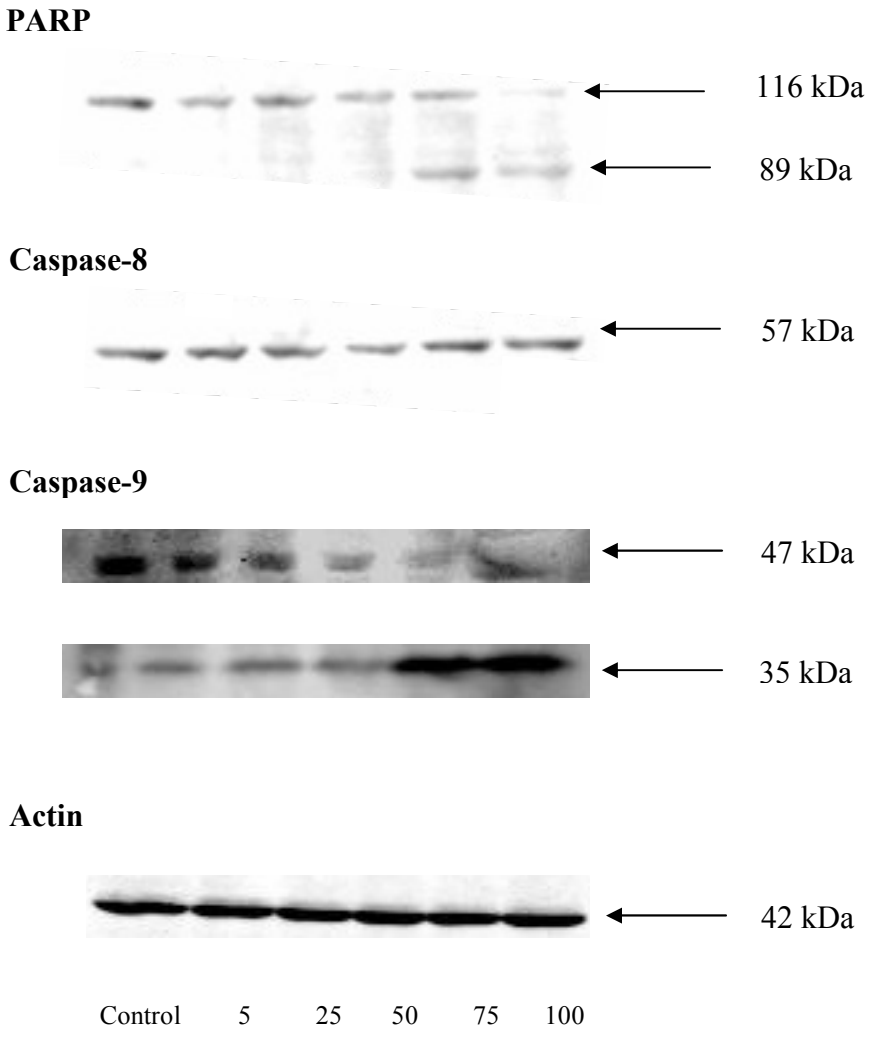
could be seen indicating that apoptosis was not taking place at the concentrations tested (upto 100 μM). 8-gingerol, on the other hand, was able to induce apoptosis at both 75 and 100 μM in LNCaP cells (Figure 60) and at 50, 75 and 100 μM in PC-3 cells (Figure 61) as seen with PARP cleavage (89 kDA cleavage product from the 116 kDa PARP protein). 8-gingerol induced cleavage of caspase-9 (35 kDa cleavage product from 47 kDa protein) in both LNCaP and PC-3 cells indicating that it acts through activation of the intrinsic pathway of apoptosis.

6-shogaol also induced apoptosis in LNCaP and PC-3 cells as seen previously. It was able to induce PARP cleavage at 100 μM in LNCaP cells and at 75 and 100 μM in PC-3 cells (Figures 62-63). As seen with 8-gingerol, it did not induce apoptosis through the extrinsic pathway (caspase 8) but through the intrinsic pathway (caspase-9).

In addition, time dependent induction of apoptosis by 8-gingerol (at 75 μM) and 6-shogaol (at 100 μM) was also conducted in both the cell lines. Both 8-gingerol and 6-shogaol were able to induce apoptosis in LNCaP cells 6 hours after treatment (as seen with caspase-9 cleavage, Figures 64-65). In PC-3 cells, treatment with 8-gingerol induced apoptosis after 6 hours (Figure 66) while treatment with 6-shogaol induced apoptosis after only 3 hours of treatment (Figure 67).

FIGURE 60

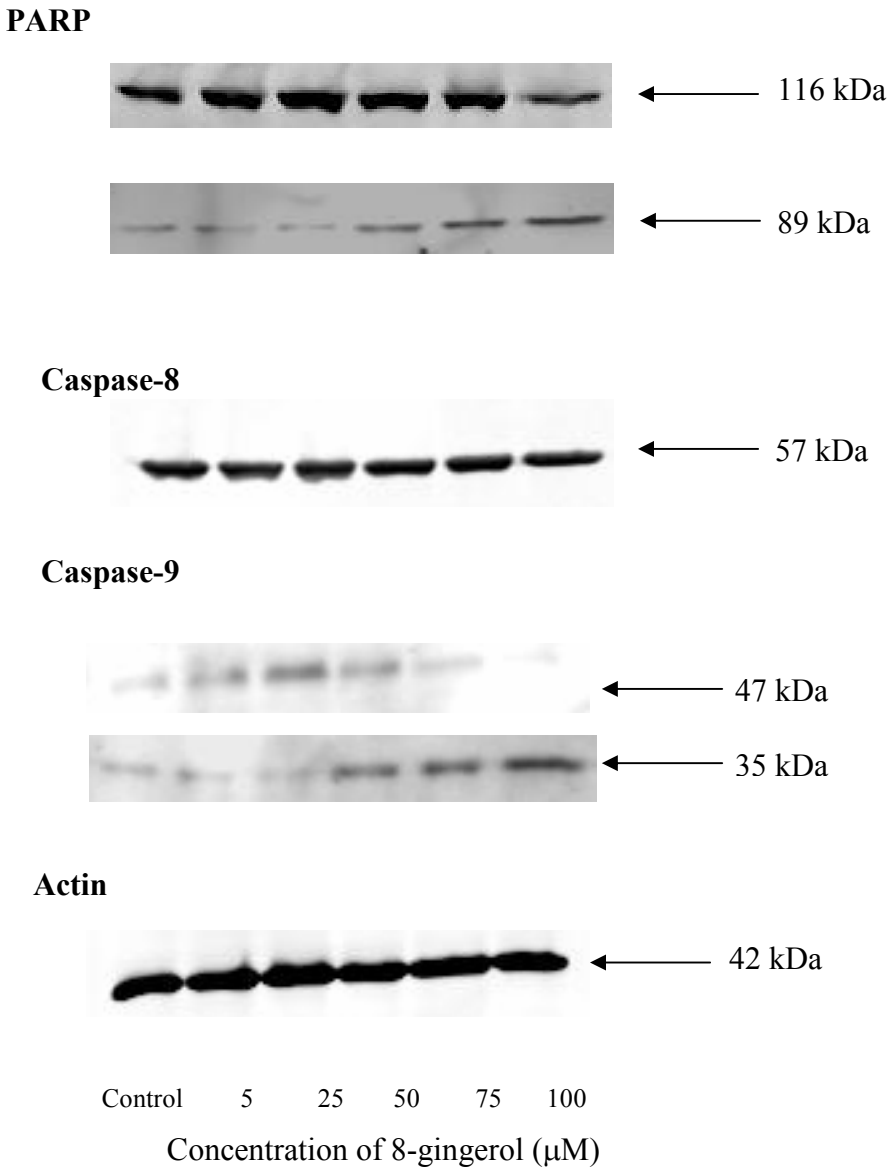
Effect of 8-gingerol on expression of apoptotic proteins in LNCaP cells



LNCaP cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 8-gingerol for 48 hours. At the end of the treatment, protein from the cells were extracted using RIPA buffer containing 1% protease inhibitor and phosphatase inhibitor cocktails, according to established protocols as described in materials and methods. The proteins in samples were determined using a BCA protein assay kit. Equal amounts (30 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The electrophoresed were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band (shown in the above figures) as described earlier.

FIGURE 61

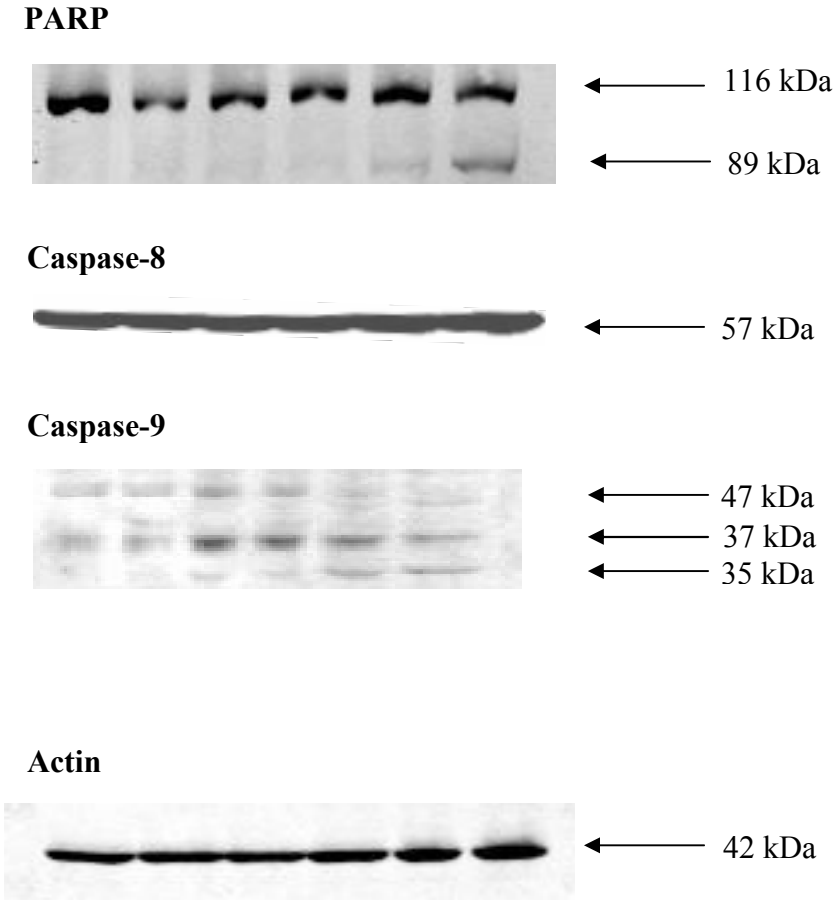
Effect of 8-gingerol on expression of apoptotic proteins in PC-3 cells



PC-3 cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 8-gingerol for 48 hours. At the end of the treatment, protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols as described in materials and methods. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The electrophoresed proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band (shown in the above figures) as described earlier.

FIGURE 62

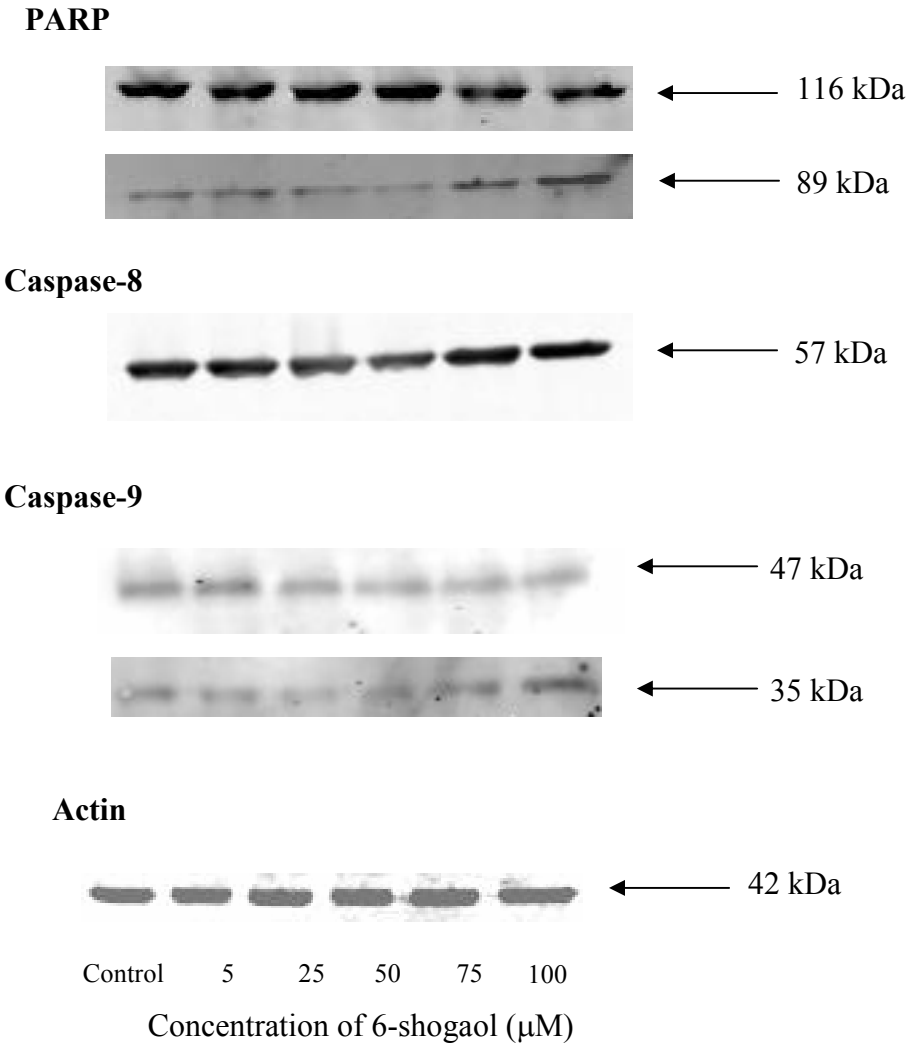
Effect of 6-shogaol on expression of apoptotic proteins in LNCaP cells



LNCaP cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 6-shogaol for 48 hours. At the end of the treatment, protein from the cells were extracted using RIPA buffer containing 1% protease inhibitor and phosphatase inhibitor cocktails, according to established protocols as described in materials and methods. The proteins in samples were determined using a BCA protein assay kit. Equal amounts (30 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The electrophoresed were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band (shown in the above figures) as described earlier.

FIGURE 63

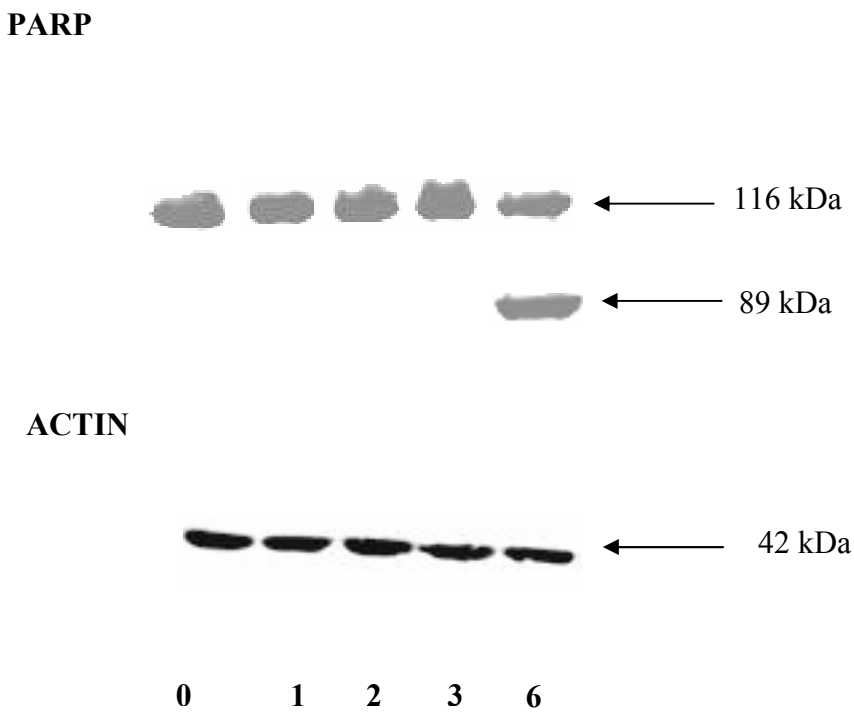
Effect of 6-shogaol on expression of apoptotic proteins in PC-3 cells



PC-3 cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with different concentrations of 6-shogaol for 48 hours. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band.

FIGURE 64

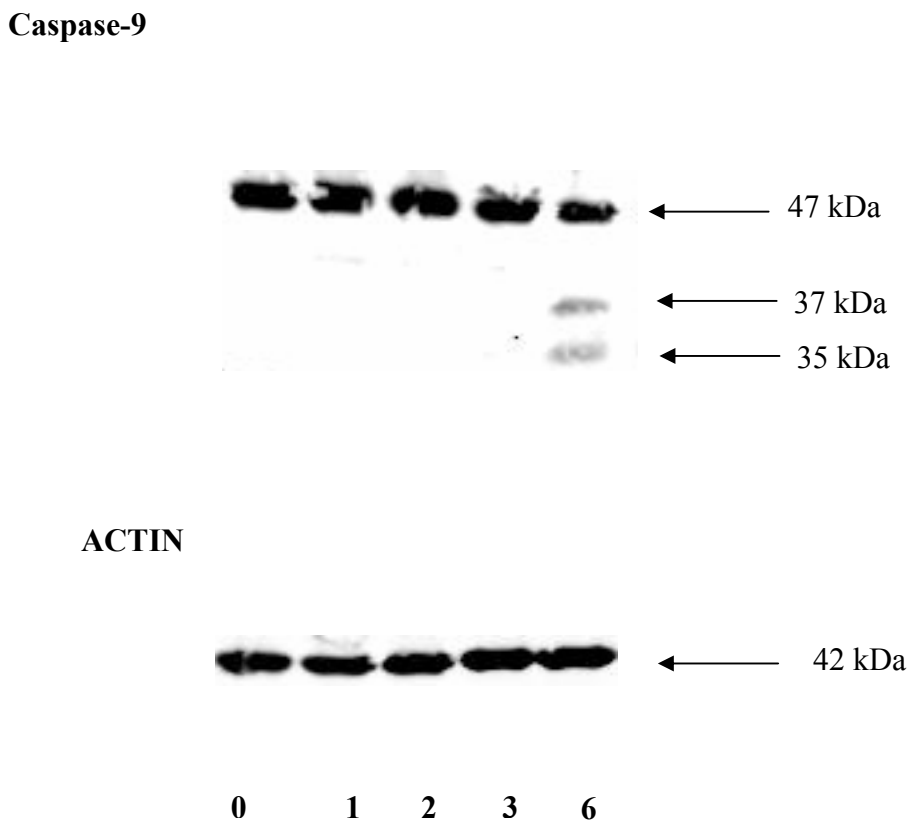
Time-dependent induction of apoptosis in LNCaP cells by treatment with 8-gingerol



LNCaP cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with 75 μM of 8-gingerol for different time periods. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band.

FIGURE 65

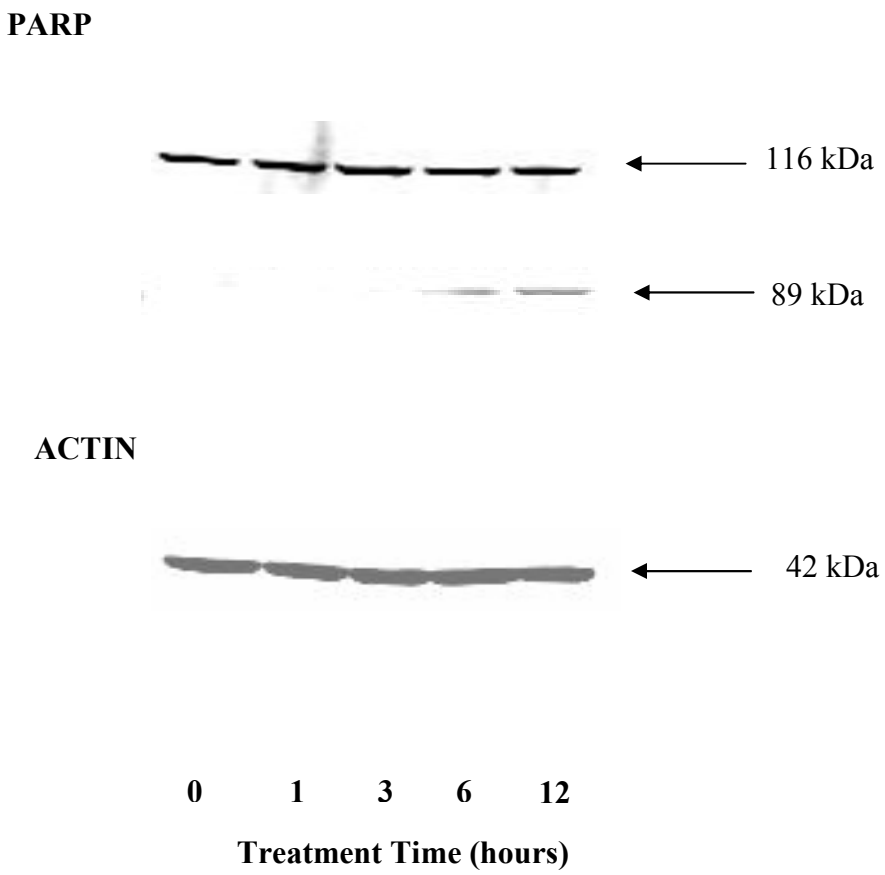
Time-dependent induction of apoptosis in LNCaP cells by treatment with 6-shogaol



LNCaP cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with 100 μM of 6-shogaol for different time periods. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band.

FIGURE 66

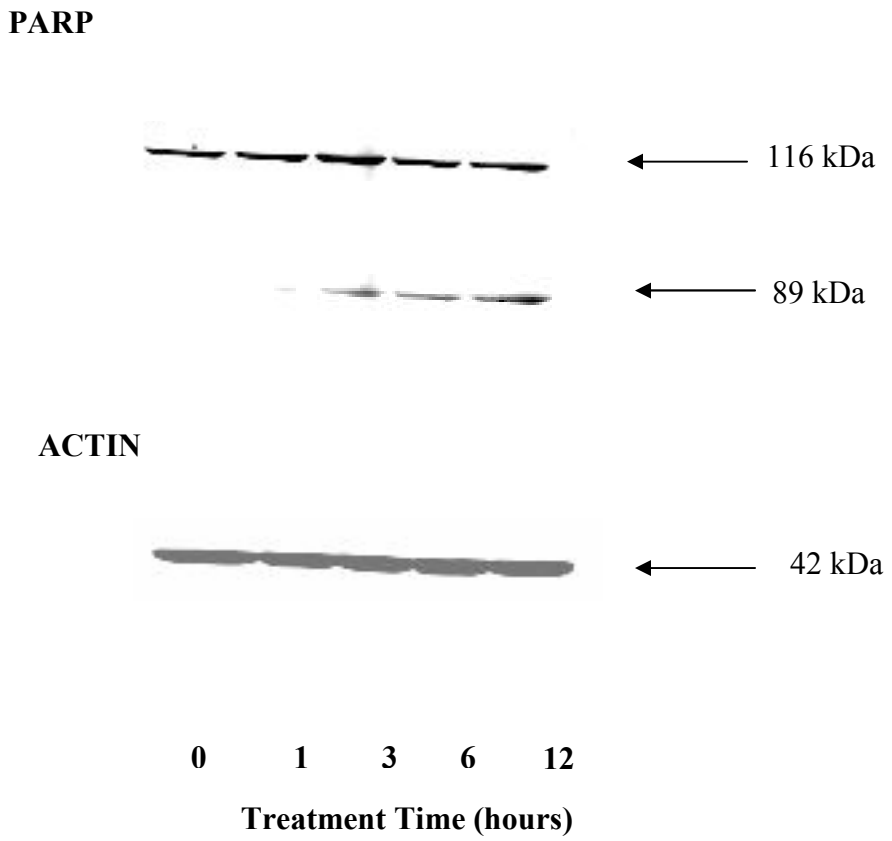
Time-dependent induction of apoptosis in PC-3 cells by treatment with 8-gingerol



PC-3 cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with 75 μM of 8-gingerol for different time periods. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols. Equal amounts (50 μg) proteins were loaded on a freshly prepared polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band.

FIGURE 67

Time-dependent induction of apoptosis in PC-3 cells by treatment with 6-shogaol



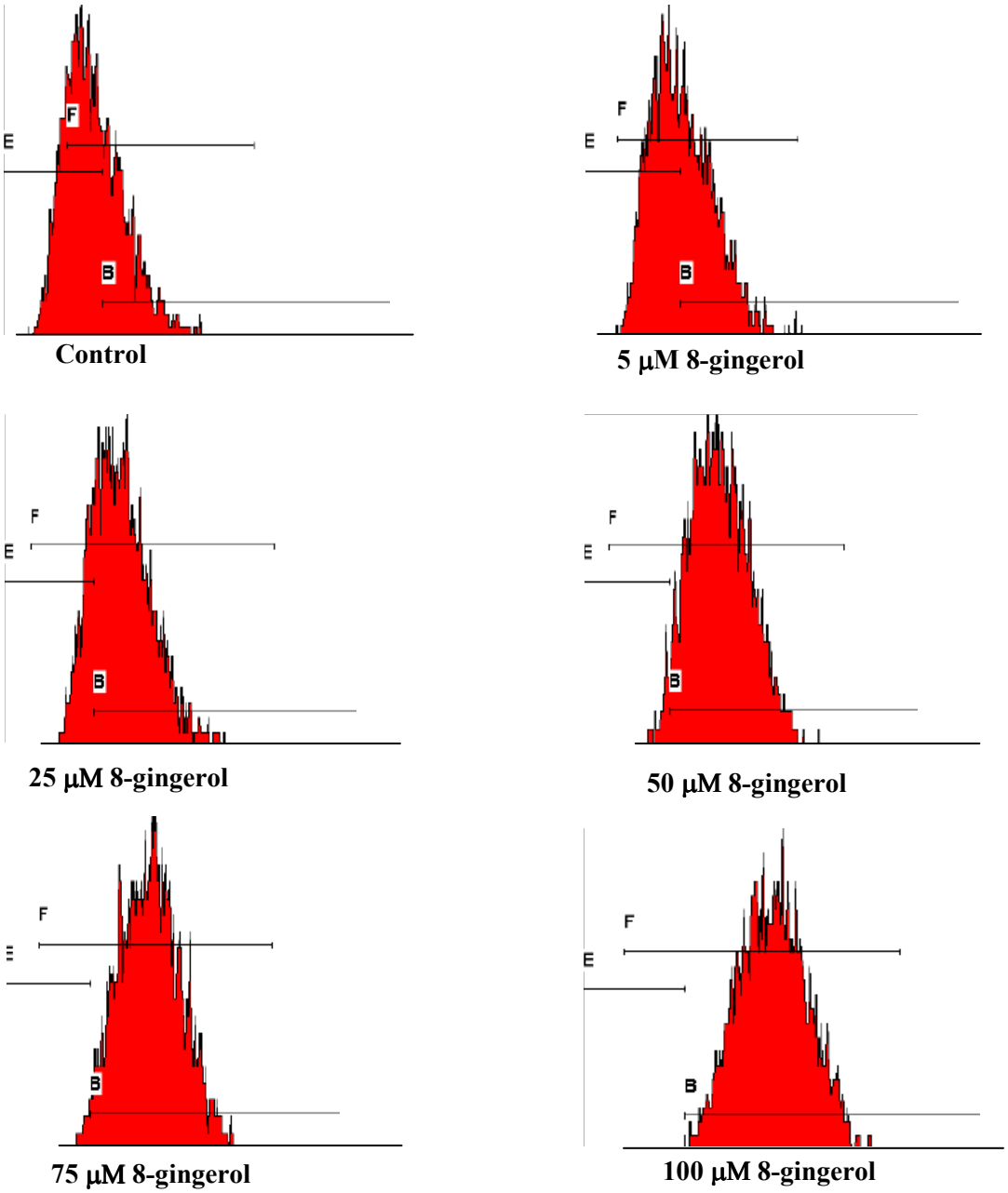
PC-3 cells were seeded at 5 x 10^5 cells/dish in 100mm^2 Petri dishes and treated with 100 μM of 6-shogaol for different time periods. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails, according to established protocols. Equal amounts (50 μg) proteins were loaded on a freshly prepared 8-12% polyacrylamide gel and electrophoresed under 70-120 V for 2-3 hours. The proteins were then transferred onto activated nitrocellulose membrane at 100 V for 2 hours. The membrane was blocked and incubated with the respective primary antibodies overnight. The appropriate secondary antibody was added and incubated for another hour. The membranes were then scanned to visualize the protein band.

Effect of 8-gingerol and 6-shogaol on reactive oxygen species (ROS) production measured using flow cytometry

The effect of 8-gingerol and 6-shogaol on ROS production was also conducted to determine the mechanism of apoptosis induction by these compounds. In short, LNCaP or PC-3 cells were pretreated with 5 mM of 2',7'-dichlorofluorescein diacetate for 15 minutes in a shaking water bath. The cells were then incubated with the respective test compounds for another hour in a shaking water bath. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp. The increase in fluorescence intensity was measured to indicate the production of reactive oxygen species. Both the test compounds were able to induce the production of ROS in both the cell lines in a dose dependent manner. 8-gingerol and 6-shogaol (at 100 μM) were able to increase the ROS levels by 7 and 8 fold respectively compared to the control in LNCaP cells (Figures 68-69). Both the test compounds were able to increase the production of ROS in PC-3 cells by 6 fold (Figures 70-71). These results correlated well with the induction of apoptosis that was seen with western blot analysis, indicating that this could be one of the mechanisms of apoptosis induction in both LNCaP and PC-3 cells.

Figure 68 a

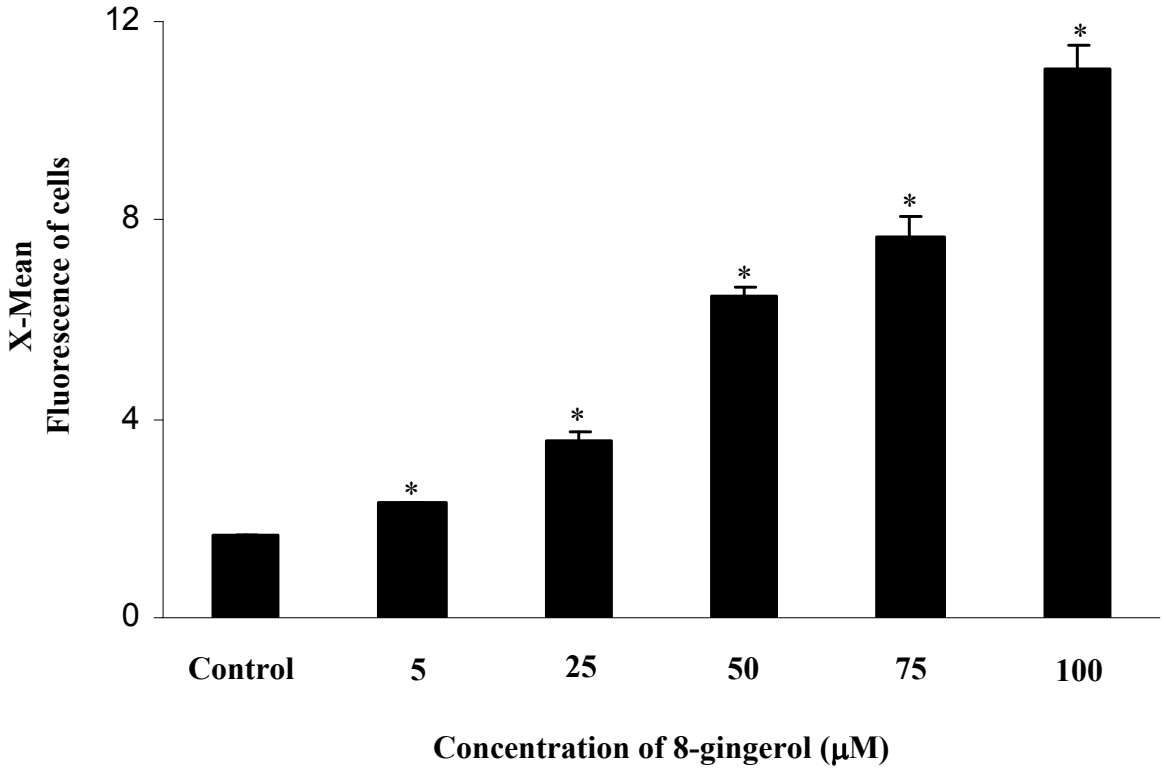
Effect of 8-gingerol on induction of ROS in LNCaP cells



LNCaP cells were treated with 5 µM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 8-gingerol (or no treatment for control) in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp.

Figure 68 b

Effect of 8-gingerol on induction of ROS in LNCaP cells

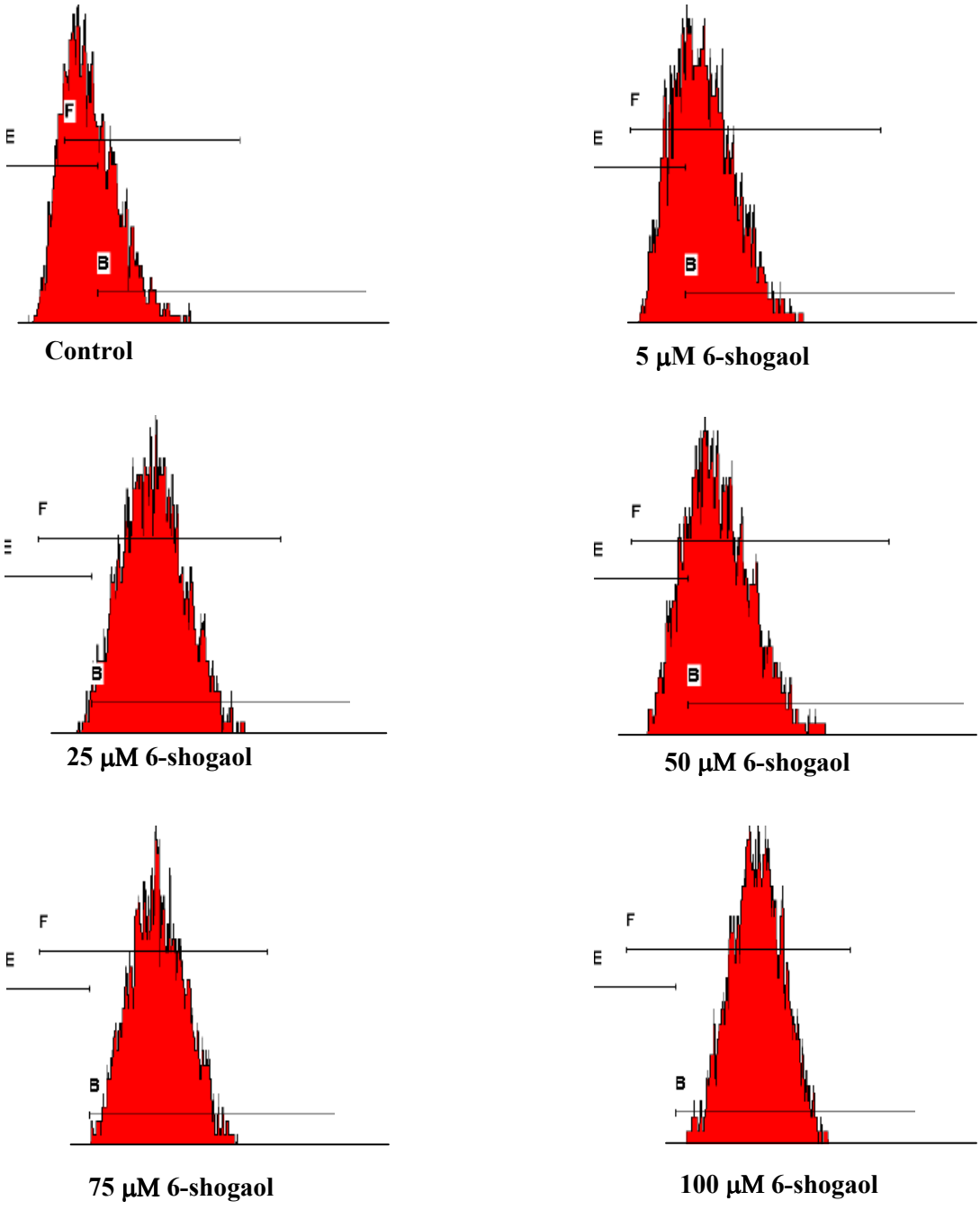


LNCaP cells were treated with 5 μM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 8-gingerol (or no treatment for control) in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp. Statistical analysis was performed using the Students *t* test.

* $p < 0.05$

Figure 69 a

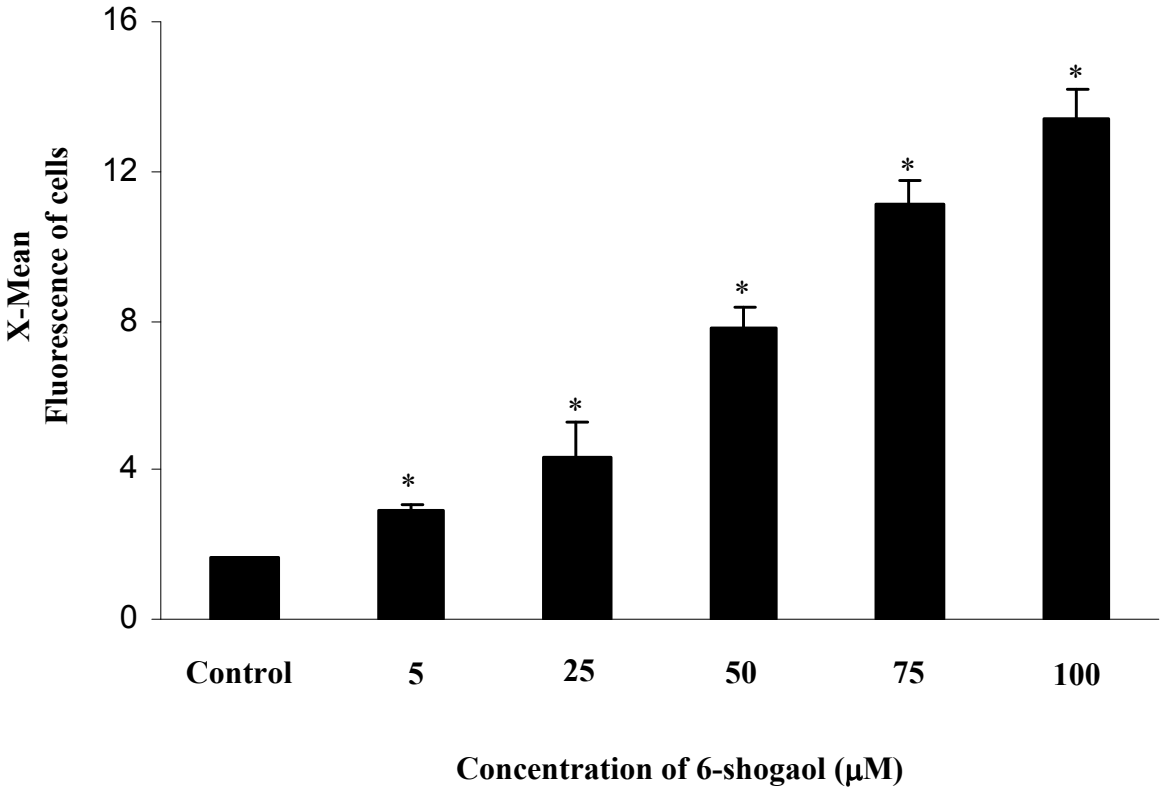
Effect of 6-shogaol on induction of ROS in LNCaP cells



LNCaP cells were treated with 5 µM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 6-shogaol (or no treatment for control) in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp.

Figure 69 b

Effect of 6-shogaol on induction of ROS in LNCaP cells

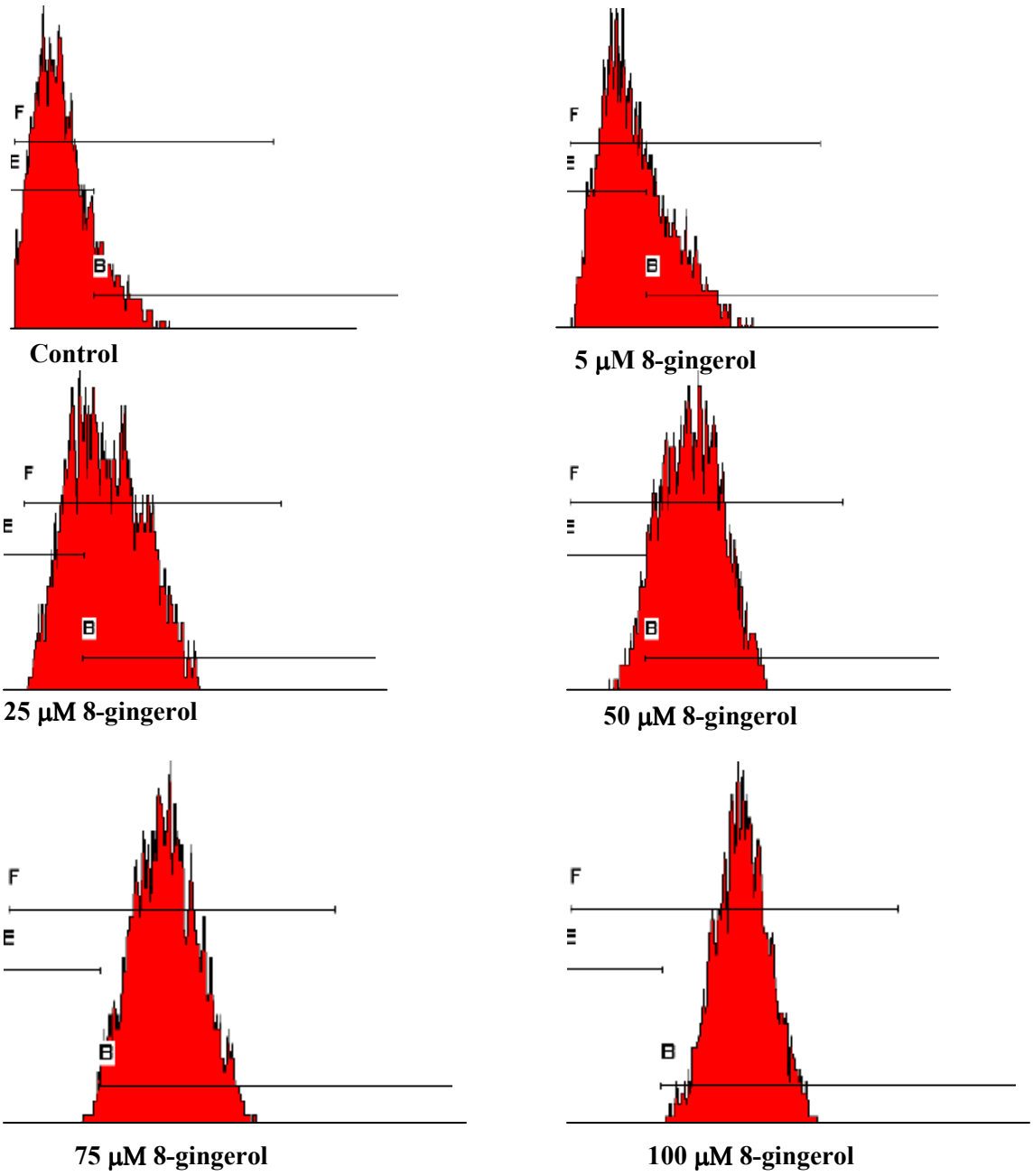


LNCaP cells were treated with 5 μM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 6-shogaol (or no treatment for control) in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was then measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp. Statistical analysis was performed using the Students *t* test.

* $p < 0.05$

Figure 70 a

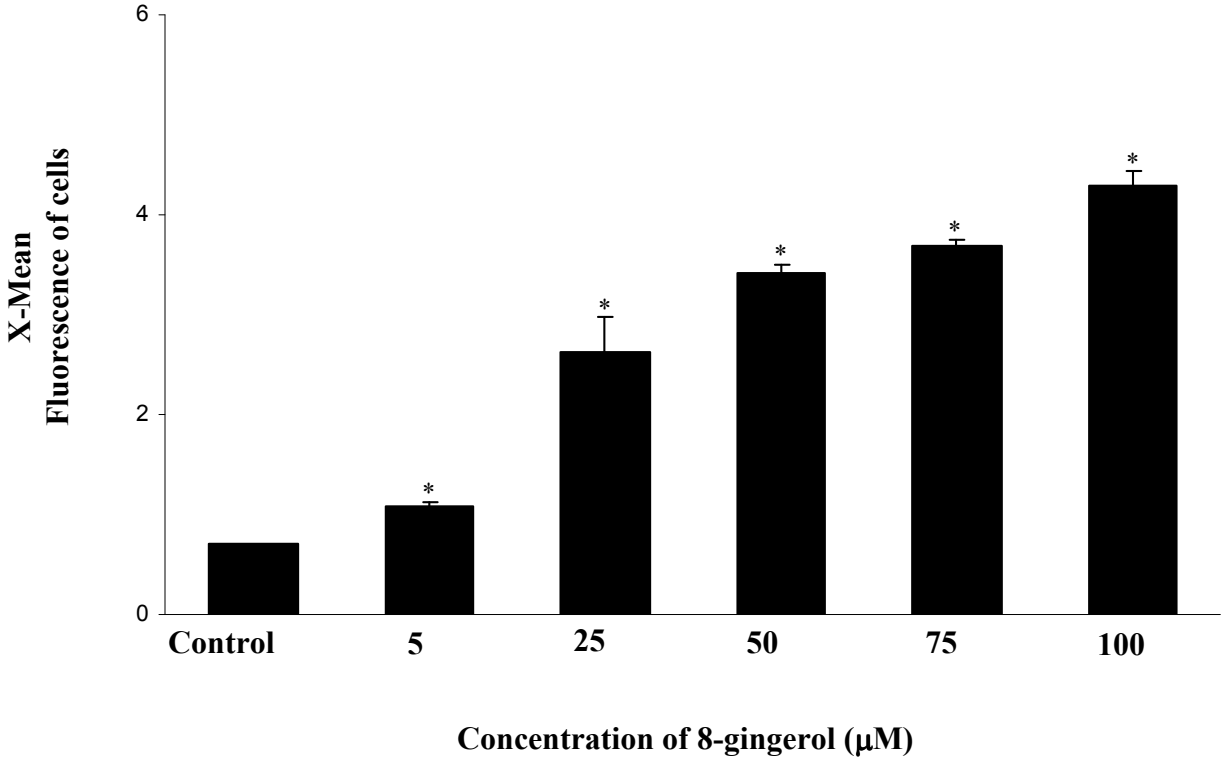
Effect of 8-gingerol on induction of ROS in PC-3 cells



PC-3 cells were treated with 5 µM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 8-gingerol in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp.

Figure 70 b

Effect of 8-gingerol on induction of ROS in PC-3 cells

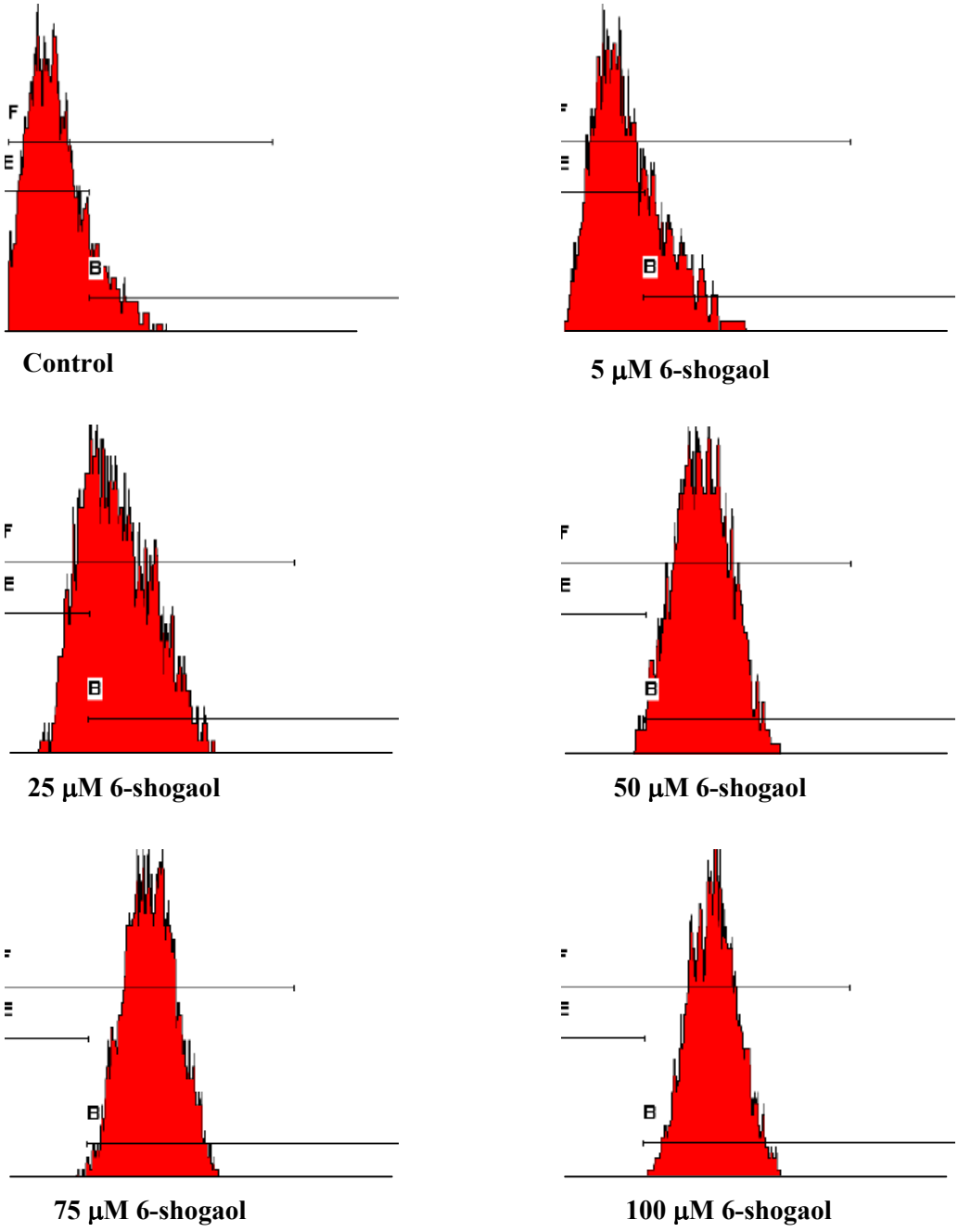


PC-3 cells were treated with 5 μM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 8-gingerol in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp. Statistical analysis was performed using the Students t test.

* $p < 0.05$

Figure 71 a

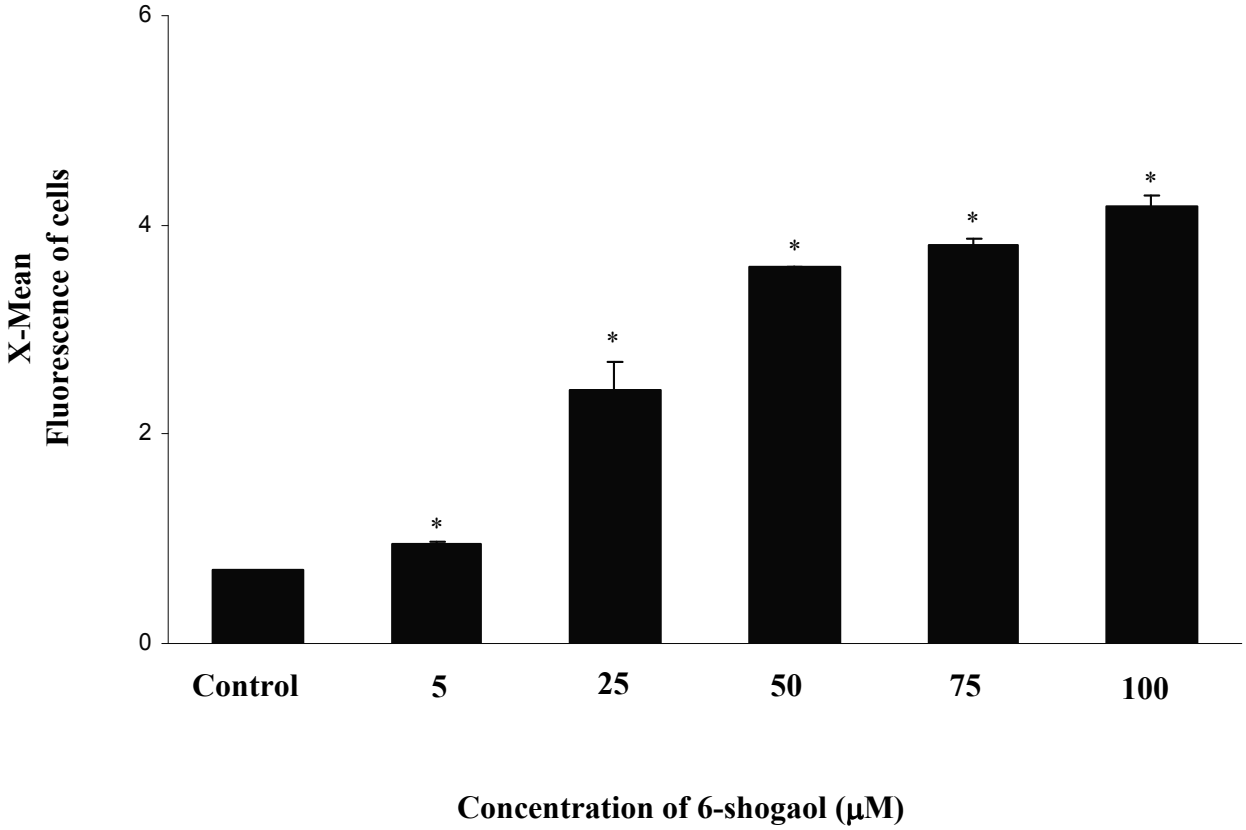
Effect of 6-shogaol on induction of ROS in PC-3 cells



PC-3 cells were treated with 5 µM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 6-shogaol in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp.

Figure 71 b

Effect of 6-shogaol on induction of ROS in PC-3 cells



PC-3 cells were treated with 5 μM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 15 minutes in a shaking water bath at 37 °C. The cells were then treated with different concentrations of 8-gingerol in triplicate and incubated for another hour at the same conditions. The fluorescence of the cells was measured using a Coulter Epics-Profile II flow cytometer. Data analysis was performed using cytologic software from Coulter Corp. Statistical analysis was performed using the Students t test.

* $p < 0.05$

DISCUSSION

Several phytochemicals isolated from fruits, vegetables, spices and herbs have shown promise as potential cancer chemopreventive agents. They have been shown to act through multiple mechanisms including modulation of the phase I and phase II drug metabolizing enzymes, scavenging of free radicals, modulation of arachidonic acid metabolism, suppression of abnormal cancer cell proliferation and induction of apoptosis [317].

Ginger has been used in traditional medicine for several ages for the treatment of a variety of disorders [274,318]. Recent studies on ginger has shown antioxidant, anti-inflammatory, chemopreventive, hypocholestrolemic and several other pharmacological activities [9,11,24-37]. The main purpose of this study was to evaluate the anti-inflammatory and chemopreventive activities of the some of the gingerols and shogaols isolated from ginger.

In this study, we have isolated a mixture of gingerols, 6-gingerol, 8-gingerol, a mixture of shogaols and 6-shogaol from a crude ginger extract using column chromatography. The purity and identity of these compounds were confirmed using HPLC and NMR analysis.

The anti-inflammatory activity of gingers has been evaluated in several *in vitro* studies and a few *in vivo* studies, mostly focusing on crude ginger and 6-gingerol. Ginger and some of its components (such as 6-gingerol, 6-paradol and related analogues) have been shown to inhibit inflammation in isolated macrophage cells. In these studies, these compounds have been know to inhibit several inflammatory markers (such as

cycloooxygenase, lipoxygenase, ornithine decarboxylase, iNOS and NF-κB) in a dose dependent manner [36,319-322]. However studies evaluating the anti-inflammatory activity of ginger and its components in animal models are very limited. Most studies have focused on the effect of crude ginger extract (ethanolic) which has been able to inhibit the edema in mice and rats in a dose dependent manner [88,106]. In a study conducted by Park et al. 6-gingerol at 10 μMol was able to reduce TPA-induced edema by 61% in female ICR mice [89].

In the current study, we wanted to evaluate the anti-inflammatory activity of 6-gingerol and 6-shogaol on the TPA-induced mouse ear inflammatory model. On application of TPA to female CD-1 mice ears, there was edema and overexpression of cytokines as noted previously. 6-gingerol was able to significantly inhibit TPA-induced ear edema in a dose dependent manner at all concentrations tested in both the experiments. However, only 25 and 50 μMol of 6-gingerol was able to inhibit the over-expression of cytokines (IL-1β and IL-6) induced by TPA. 6-shogaol (25 and 50 μMol) was also able to inhibit ear edema and over expression of cytokines in a dose dependent manner. Further studies are warranted to establish the effect of these compounds on other important pathways involved in inflammation.

The main purpose of thus study was to evaluate the chemopreventive activity of these isolated compounds on prostate cancer cells. The chemopreventive activity of ginger has been evaluated in a few *in vitro* and *in vivo* experiments, primarily focusing on crude ginger and 6-gingerol. Ginger extract has been shown to inhibit cancers of the colon, skin, breast and liver in animal models [30,86,323-325]. Ginger extract has also been shown to possess anti-oxidant potential in several *in vitro* experiments which could

contribute to its chemopreventive effect [27,34,58,104,326]. In addition 6-gingerol has been shown to inhibit angiogenesis in mice and in certain human cell lines [99]. Ginger and 6-gingerol has been shown to induce apoptosis in leukemia, oral and skin cancer cell lines [78,97,327,328].

In this study the effects of gingerols and shogaols on prostate cancer cells were evalauted. We selected two different prostate cancer cells lines (LNCaP and PC-3) to study the chemopreventive potential of these compounds, an androgen dependent (LNCaP) and an androgen independent (PC-3) cell line. In order to evaluate our hypotheses, we first conducted cell viability studies using the MTT assay. By this method we were able to establish the IC_{50} (the concentration that was able to inhibit cell growth and viability by 50%) of the compounds. The test compounds were able to inhibit the growth and viability of both prostate cancer cell lines, with 8-gingerol and 6-shogaol being the most effective in both cell lines. However, the androgen dependent, LNCaP cells were more sensitive and a lower dose of the test compounds were able to inhibit cell viability and proliferation. Based on this we conducted several assays to establish the mechanisms of action of the test compounds on apoptosis.

In order to detect the presence of apoptosis, we first conducted experiments to study the morphological changes occurring in the cells (that are characteristic of apoptosis) on treatment with the test compounds. This was conducted with the help of fluorescence microscopy, using propidium iodide as the dye. We could observe most of the morphological features of apoptosis such as fragmented nuclei, chromatin condensation and the presence of apoptotic bodies. As observed with the MTT assay, 8-

gingerol, shogaol mixture and 6-shogaol were most effective in inducing apoptosis in both cell lines.

To confirm the presence of apoptosis in treated cells, we performed cell cycle analysis and Annexin V-FITC and PI co-staining. The cell cycle analysis was conducted using PI staining and the extent of staining in each phase of the cell cycle was measured using flow cytometry as described previously. The effects of the test compounds on different phases of the cell cycle were very different indicating that they might act through different mechanisms. 6-gingerol was able to induce apoptosis, as seen with subG1 phase arrest, only at 100 μM in LNCaP cells. However it did induce G2M phase arrest in PC-3 cells. 8-gingerol was able to induce apoptosis at 75 and 100 μM in LNCaP cells but not on any other phases of the cell cycle. On the other hand, 8-gingerol was able to induce both subG1 (at 100 μM) and S phase arrest (at 75 and 100 μM) in PC-3 cells.

6-shogaol was able to induce apoptosis at 75 and 100 μM and S phase arrest at 75 μM in LNCaP cells. In PC-3 cells 6-shogaol was able to induce apoptosis from 25 μM onwards as seen with subG1 arrest. In addition, it was also able to induce S phase arrest in PC-3 cells from 50 μM. However the extent of apoptosis was less in PC-3 cells than LNCaP as noted previously.

A recent study using p53 mutant pancreatic cell lines have shown that 6-gingerol at 400 μM induces G1 and S phase arrest in the cell cycle [91]. However, this concentration may not be achievable in humans as the bioavailability of most phytochemicals is low. Previous studies have also shown that on intravenous administration to rats, 6-gingerol is rapidly cleared from the blood with less than 0.1% of the dose remaining in the plasma after 30 minutes [116]. Hence, we have used a highest

concentration of 100 μM in our study, which might be relevant to the human situation. Detailed mechanistic studies are warranted to further evaluate the exact mechanisms of action of these compounds.

In addition, Annexin V- FITC analysis was performed to confirm the presence of apoptosis. During the process of apoptosis, externalization of phosphotidyl serine (PS) (an amino phospholipid normally located in the inner portion of the cell membrane) occurs, aiding in its easy recognition by macrophages and other phagocytes. This phenomenon which is an early event during apoptosis, is used in this assay to determine the presence of apoptosis. Both 8-gingerol and 6-shogaol were able to induce apoptosis in LNCaP and PC-3 cells based on this assay. This data correlates well with the results discussed previously in this study.

To further elucidate the pathways of apoptosis induction by the test compounds, Western blot analysis was performed. Both, 8-gingerol and 6-shogaol induced apoptosis in LNCaP and PC-3 cells as seen with PARP cleavage. To further detect the mechanisms of apoptosis induction, caspase-8 and caspase-9 Western blots were conducted. Both, 8-gingerol and 6-shogaol, induced apoptosis through the intrinsic or mitochondrial pathway as seen with caspase-9 cleavage in both LNCaP and PC-3 cell lines. To further elucidate the mechanisms of apoptosis induction, we determined the levels of ROS in 8-gingerol and 6-shogaol treated cells. Both 8-gingerol and 6-shogaol were able to induce the production of ROS in LNCaP and PC-3 cells in a dose dependent manner which correlated well with induction of apoptosis. This indicated that production of reactive oxygen species such as hydrogen peroxide could be one of the mechanism through which these compounds exert their chemopreventive and apoptotic effect in prostate cancer cells.

In this study, we have shown for the first time that both 8-gingerol and 6-shogaol were able to induce apoptosis in androgen dependent (LNCaP) and androgen independent (PC-3) cells through activation of the intrinsic or mitochondrial pathway of apoptosis.

REFERENCES

1. Balladin, DA, Headley, O, Chang-Yen, I and McGaw, DR (1998) High pressure liquid chromatographic analysis of the main pungent principles of solar dried West Indian ginger (Zingiber officinale Roscoe). *Renewable Energy*, **13**, 531-536.
2. Azian, MN, Kamal, AA M and Azlina, MN (2004) Changes of cell structure in ginger during processing. *Journal of Food Engineering*, **62**, 359-364.
3. Young, HY, Chiang, CT, Huang, YL, Pan, FP and Chen, GL (2002) Analytical and stability studies of ginger preparations. *Journal of Food and Drug Analysis*, **10**, 149-153.
4. Zarate, R and Yeoman, MM (1996) Changes in the amounts of [6]gingerol and derivatives during a culture cycle of ginger, Zingiber officinale. *Plant Science*, **121**, 115-122.
5. Bartley, JP and Jacobs, AL (2000) Effects of drying on flavour compounds in Australian-grown ginger (Zingiber officinale). *Journal of the Science of Food and Agriculture*, **80**, 209-215.
6. Ravindran, PN and Babu, KN (2004) Introduction. In Babu, K. (ed.), *Ginger the genus Zingiber*. CRC Press, Boca Raton, FL, USA, pp. 1-14.
7. Wu, TS, Wu, WC, Wu, PL, Chern, CY, Leu, YL and Chan, YY (1998) Structure and synthesis of [n]-dehydroshaosaols from Zingiber officinale. *Phytochemistry*, **48**, 889-891.
8. Grant, K. L. and Lutz, R. B. (2000) Ginger. *Am J Health Syst Pharm*, **57**, 945-947.
9. Kimura, I, Pancho, LR and Tsuneki, H (2004) Pharmacology of ginger. In Babu, K. (ed.), *Ginger: The genus zingiber*. CRC Press, Boca Raton, FL, USA, pp. 469-488.
10. Kulkarni, A (2004) Ayurved for digestion. *Complementary health care practices*, **23**, 128-129.
11. Mustafa, T, Srivastava, KC and Jensen, KB (1993) Drug development report (9): Pharmacology of ginger, *Zingiber officinale*. *Journal of drug development*, **6**, 25-39.
12. Balladin, DA and Headley, O (1999) Liquid chromatographic analysis of the main pungent principles of solar dried West Indian ginger (Zingiber officinale Roscoe). *Renewable Energy*, **18**, 257-261.
13. Connell, DW and Sutherland, MD (1969) A re-examination of gingerol, shogaol, and zingerone, the pungent principles of ginger (*Zingiber officinale* roscoe). *Australian journal of chemistry*, **22**, 1033-1043.
14. Variyar, PS, Gholap, AS and Thomas, P (2000) Estimation of pungency in fresh gingers: A new fluorimetric assay. *Journal of Food Composition and Analysis*, **13**, 219-225.

15. Bhattarai, S., Tran, VH and Duke, CC (2001) The stability of gingerol and shogaol in aqueous solutions. *Journal of Pharmaceutical Sciences*, **90**, 1658-64.

16. Balladin, D, Headley, O, Chang-Yen, I and McGaw, D (1997) Extraction and evaluation of the main pungent principles of solar dried West Indian ginger (Zingiber officinale ROSCOE) rhizome. *Renewable energy*, **12**, 125-130.

17. Vernin, G and Parkanya, C (2004) Chemistry of ginger. In Babu, K. (ed.), *Ginger: The genus zingiber*. CRC Press, Boca Raton, FL, USA, pp. 87-180.

18. Kikuzaki, H and Nakatani, N (1996) Cyclic diarylheptanoids from rhizomes of Zingiber officinale. *Phytochemistry*, **43**, 273-277.

19. Hori, Y, Miura, T, Hirai, Y, Fukumura, M, Nemoto, Y, Toriizuka, K and Ida, Y (2003) Pharmacognostic studies on ginger and related drugs--part 1: five sulfonated compounds from Zingiberis rhizome (Shokyo). *Phytochemistry*, **62**, 613-617.

20. Ma, J, Jin, X, Yang, L and Liu, ZL (2004) Diarylheptanoids from the rhizomes of Zingiber officinale. *Phytochemistry*, **65**, 1137-1143.

21. Gong, F, Fung, YS and Liang, YZ (2004) Determination of volatile components of ginger using gas chromatography-mass spectrometry with resolution improved by data processing techniques. *Journal of Agricultural and Food Chemistry*, **52**, 6378-6383.

22. Nishimura, O (1995) Identification of the Characteristic Odorants in Fresh Rhizomes of Ginger (Zingiber-Officinale Roscoe) Using Aroma Extract Dilution Analysis and Modified Multidimensional Gas-Chromatography Mass-Spectroscopy. *Journal of Agricultural and Food Chemistry*, **43**, 2941-2945.

23. Onyenekwe, PC and Hashimoto, S (1999) The composition of the essential oil of dried Nigerian ginger (*Zingiber officinale* Roscoe). *European Food Research Technology*, **209**, 407-410.

24. Ahmed, RS, Seth, V, Pasha, ST and Banerjee, BD (2000) Influence of dietary ginger (Zingiber officinales Rosc) on oxidative stress induced by malathion in rats. *Food Chemical Toxicology*, **38**, 443-50.

25. Borrelli, F, Capasso, R, Pinto, A and Izzo, AA (2004) Inhibitory effect of ginger (Zingiber officinale) on rat ileal motility in vitro. *Life Sciences*, **74**, 2889-96.

26. Young, HY, Luo, YL, Cheng, HY, Hsieh, WC, Liao, JC and Peng, WH (2005) Analgesic and anti-inflammatory activities of [6]-gingerol. *Journal of ethnopharmacology*, **96**, 207-210.

27. Zia-ur-Rehman, Salariya, A. M. and Habib, F. (2003) Antioxidant activity of ginger extract in sunflower oil. *Journal of the Science of Food and Agriculture*, **83**, 624-629.

28. Yamahara, J , Mochizuki, M , Rong, HQ, Matsuda, H and Fujimura, H. (1988) The anti-ulcer effect in rats of ginger constituents. *Journal of Ethnopharmacology*, **23**, 299-304.

29. Tanabe, M, Chen, Y-D, Saito, K-I and Kano, Y (1993) Cholesterol biosynthesis inhibitory component from *Zingiber officinale* Roscoe. *Chemical and Pharmaceutical bulletin*, **41**, 710-713.

30. Surh, YJ, Park, KK, Chun, KS, Lee, JM, Lee, SS and Lee, SS (1999) Anti-tumor promoting activities of selected pungent phenolic substances present in ginger. *Journal of Enviromnemtal pathology, toxicology and oncology*, **18**, 131-139.

31. Schuhbaum, H, Burgerneister, J, Paper, DH and Franz, G (2000) Anti-inflammatory activity of Zingiber officinale extracts. *Pharmaceutical and Pharmacological letters*, **2**, 82-85.

32. Park, KK, Chun, KS, Lee, JM, Lee, SS and Surh, YJ (1998) Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice. *Cancer Letters*, **129**, 139-144.

33. Nurtjahja-Tjendraputra, E, Ammit, AJ, Roufogalis, BD, Tran, VH and Duke, CC (2003) Effective anti-platelet and COX-1 enzyme inhibitors from pungent constituents of ginger. *Thrombosis Research*, **111**, 259-265.

34. Masuda, Y, Kikuzaki, H, Hisamoto, M and Nakatani, N (2004) Antioxidant properties of gingerol related compounds from ginger. *Biofactors*, **21**, 293-296.

35. Lee, E and Surh, YJ (1998) Induction of apoptosis in HL-60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. *Cancer Letters*, **134**, 163-168.

36. Ippoushi, K, Azuma, K, Ito, H, Horie, H and Higashio, H (2003) [6]-Gingerol inhibits nitric oxide synthesis in activated J774.1 mouse macrophages and prevents peroxynitrite-induced oxidation and nitration reactions. *Life Sciences*, **73**, 3427-3437.

37. Frondoza, CG, Sohrabi, A, Polotsky, A, Phan, PV, Hungerford, DS and Lindmark, L (2004) An in vitro screening assay for inhibitors of proinflammatory mediators in herbal extracts using human synoviocyte cultures. *In Vitro Cellular and Developmental Biology Animal*, **40**, 95-101.

38. Agarwal, M., Walia, S., Dhingra, S. and Khambay, B. P. (2001) Insect growth inhibition, antifeedant and antifungal activity of compounds isolated/derived from Zingiber officinale Roscoe (ginger) rhizomes. *Pest Manag Sci*, **57**, 289-300.

39. Ficker, C., Smith, M. L., Akpagana, K., Gbeassor, M., Zhang, J., Durst, T., Assabgui, R. and Arnason, J. T. (2003) Bioassay-guided isolation and identification of antifungal compounds from ginger. *Phytother Res*, **17**, 897-902.

40. Ficker, C. E., Arnason, J. T., Vindas, P. S., Alvarez, L. P., Akpagana, K., Gbeassor, M., De Souza, C. and Smith, M. L. (2003) Inhibition of human pathogenic fungi by ethnobotanically selected plant extracts. *Mycoses*, **46**, 29-37.

41. Ficker, C. E., Smith, M. L., Susiarti, S., Leaman, D. J., Irawati, C. and Arnason, J. T. (2003) Inhibition of human pathogenic fungi by members of Zingiberaceae used by the Kenyah (Indonesian Borneo). *J Ethnopharmacol*, **85**, 289-293.

42. Hashimoto, K, Satoh, K, Murata, P, Makino, B, Sakakibara, I, Kase, Y, Ishige, A, Higuchi, M and Sasaki, H (2002) Component of Zingiber officinale that improves the enhancement of small intestinal transport. *Planta medica*, **68**, 936-939.

43. Satoh, K, Kase, Y, Hayakawa, T, Murata, P, Ishige, A and Sasaki, H (2001) Dai-kenchu-to enhances accelerated small intestine transport. *Biological pharmaceutical bulletin*, **24**, 1122-1126.

44. Niijima, A, Kubo, M, Hashimoto, K, Komatsu, Y, Maruno, M and Okada, M (1998) Effect of oral administration of Pinellia ternata, Zingiberis rhizoma and their mixture on the efferent activity of the gastric branch of the vagus nerve in the rat. *Neuriscience letters*, **258**, 5-8.

45. Platel, K and Srinivasan, K (2000) Influence of dietary spices and their active principles on pancreatic digestive system in albino rats. *Nahrung*, **44**, 42-46.

46. Someya, A, Horie, S, Yamamoto, H and Murayama, T (2003) Modification of capsaicin-sensitive neurons in isolated guinea pig ileum by 6-gingerol and lafutidine. *Journal of Pharmacological science*, **92**, 359-366.

47. Bhandari, U, Shamsher, AA, Pillai, KK and Khan, MSY (2003) Antihepatotoxic activity of ginger ethanol extract in rats. *Pharmaceutical Biology*, **41**, 68-71.

48. Bryer, E (2005) A literature review of the effectiveness of ginger in alleviating mild to moderate nausea and vomiting of pregnancy. *Journal of midwifery and womens health*, **50**, e1-e3.

49. Grontved, A, Brask, T, Kambskard, J and Hentzer, E (1988) Ginger root against sea sickness. *Acta Otolaryngology*, **105**, 45-49.

50. Fischer-Rasmussen, W, Kjaer, SK, Dahl, C and Asping, U (1990) Ginger treatment of hyperemesis gravidarum. *European journal of gynecology and reproductive biology*, **38**, 19-24.

51. Portnoi, G., Chng, L., Karimi-Tabesh, L., Koren, G., Tan, M. P. and Einarson, A. (2004) Prospective comparative study of the safety and effectiveness of ginger for the treatment of nausea and vomiting in pregnancy. (vol 189, pg 1374, 2003). *American Journal of Obstetrics and Gynecology*, **190**, 1140-1140.

52. Vutyavanich, T., Kraisarin, T. and Ruangsri, R. (2001) Ginger for nausea and vomiting in pregnancy: randomized, double-masked, placebo-controlled trial. *Obstet Gynecol*, **97**, 577-582.

53. Vutyavanich, T., Kraisarin, T. and Ruangsri, RA. (2001) Is ginger root effective for decreasing the severity of nausea and vomiting in early pregnancy. *The journal of family practice*, **50**, 720.

54. Westfall, R. E. (2004) Use of anti-emetic herbs in pregnancy: women's choices, and the question of safety and efficacy. *Complement Ther Nurs Midwifery*, **10**, 30-36.

55. Willetts, K. E., Ekangaki, A. and Eden, J. A. (2003) Effect of a ginger extract on pregnancy-induced nausea: A randomised controlled trial. *Australian & New Zealand Journal of Obstetrics & Gynaecology*, **43**, 139-144.

56. Vicedo, TB and Correas, FJH (2003) Antioxidants as cancer therapies. In Watson, R. (ed.), *Functional foods and nutraceuticals in cancer prevention*. Iowa state press, Ames, Iowa, pp. 3-26.

57. Aruoma, OI, Spencer, JPE, Warren, D, Jenner, P, Butler, J and Halliwell, B (1997) Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. *Food Chemistry*, **60**, 149-156.

58. Jitoe, A, Masuda, T, Tengah, IGP, Suprapta, DN, Gara, IW and Nakatani, N (1992) Antioxidant activity of tropical ginger extracts and analysis of the contained curcuminoids. *Journal of Agricultural and Food Chemistry*, **40**, 1337-1340.

59. Masuda, T and Jitoe, A (1994) Antioxidative and anti-inflammatory compounds from tropical gingers: Isolation, structure determination and activities of cassumunins A, B, and C, new complex curcuminoids from *Zingiber cassumunar*. *Journal of Agricultural and Food Chemistry*, **42**, 1850-1856.

60. Dang, MN, Takacsova, M, Nguyen, DV and Kristianova, K (2001) Antioxidant activity of essential oils from various spices. *Nahrung*, **45**, 64-66.

61. Halvorsen, BL, Holte, K, Myhrstad, MCW, Barikmo, I, Hvattum, E, Remberg, SF, Wold, AB, Haffner, K, Baugerod, H, Anderson, LF, Moskaug, JO, Jacobs, DR and Blomhoff, R (2002) A systematic screening of total antioxidants in dietary plants. *Journal of Nutrition*, **132**, 461-471.

62. Murcia, MA, Egea, I, Romojaro, F, Parras, P, Jimenez, AM and Martinez-Tome, M (2004) Antioxidant evaluation in dessert spices compared with common food additives. Influence of irradiation procedure. *Journal of Agricultural and Food Chemistry*, **52**, 1872-1881.

63. Jagetia, GC, Baliga, MS, Venkatesh, P and Ulloor, JN (2003) Influence of ginger rhizome (Zingiber officinale Rosc) on survival, glutathione and lipid peroxidation in mice after whole-body exposure to gamma radiation. *Radiat Research*, **160**, 584-592.

64. Backon, J (1986) Ginger: Inhibition of thromboxane synthetase and stimulation of prostacyclin: Relevance for medicine and psychiatry. *Medical Hypothesis*, **20**, 271-278.

65. Jolad, SD, Lantz, RC, Solyom, AM, Chen, GJ, Bates, RB and Timmermann, BN (2004) Fresh organically grown ginger (Zingiber officinale): composition and effects on LPS-induced PGE2 production. *Phytochemistry*, **65**, 1937-1954.

66. Shen, C. L., Hong, K. J. and Kim, S. W. (2003) Effects of ginger (*Zingiber officinale* Rosc.) on decreasing the production of inflammatory mediators in sow osteoarthritic cartilage explants. *Journal of Medicinal food*, **6**, 323-328.

67. Verma, S. K., Singh, J., Khamesra, R. and Bordia, A. (1993) Effect of ginger on platelet aggregation in man. *Indian J Med Res*, **98**, 240-242.

68. Kiuchi, F., Shibuya, M. and Sankawa, U. (1982) Inhibitors of prostaglandin biosynthesis from ginger. *Chem Pharm Bull (Tokyo)*, **30**, 754-757.

69. Kiuchi, F., Iwakami, S, Shibuya, M., Hanaoka, F and Sankawa, U. (1992) Inhibition of prostaglandin and leukotriene biosynthesis by gingerols and diarylheptanoids. *Chemical and Pharmaceutical bulletin*, **40**, 387-391.

70. Koo, K, Ammit, A, Tran, V, Duke, C and Roufogalis, B (2001) Gingerols and Related Analogues Inhibit Arachidonic Acid-Induced Human Platelet Serotonin Release and Aggregation. *Thrombosis Research*, **103**, 387-397.

71. Kim, SO, Chun, KS, Kundu, JK and Surh, YJ (2004) Inhibitory effects of [6]-gingerol on PMA-induced COX-2 expression and activation of NF-κB and p38 MAPK in mouse skin. *Biofactors*, **21**, 27-31.

72. The Arthritis Foundation.

73. Chapkin, R, McMurray, D and Jolly, C (2000) Dietary n-3 polyunsaturated fatty acids modulate T-lymphocyte activation: Clinical relevance in treating diseases of chronic inflammation. In Keen, C. (ed.), *Nutrition and imunology*. Humana Press, Totowa, NJ, pp. 121-134.

74. Altman, RD and Marcussen, KC (2001) Effects of a ginger extract on knee pain in patients with osteoarthritis. *Arthritis and Rheumatology*, **44**, 2531-2538.

75. Levy, A. S., Simon, O., Shelly, J. and Gardener, M. (2006) 6-Shogaol reduced chronic inflammatory response in the knees of rats treated with complete Freund's adjuvant. *BMC Pharmacol*, **6**, 12.

76. Imanishi, N., Mantani, N., Sakai, S., Sato, M., Katada, Y., Ueda, K., Terasawa, K. and Ochiai, H. (2004) Inducible activity of ginger rhizome (Zingiber officinale rosc.) on the mRNA expression of macrophage-inducible nitric oxide (NO) synthase and NO production in a macrophage cell line, RAW264.7. *American Journal of Chinese Medicine*, **32**, 727-735.

77. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C. and Thun, M. J. (2006) Cancer statistics, 2006. *CA Cancer J Clin*, **56**, 106-130.

78. Bode, AM, Ma, WY, Surh, YJ and Dong, Z (2001) Inhibition of epidermal growth factor-induced cell transformation and activator protein-1 activation by [6]-gingerol. *Cancer Research*, **61**, 850-853.

79. Surh, YJ (2002) Anti-tumor promoting potential of selected spice ingredients with anti-oxidative and anti-inflammatory activities: a short review. *Food and Chemical Toxicology*, **40**, 1091-1097.

80. Surh, YJ, Lee, E and Lee, JM (1998) Chemoprotective properties of some pungent ingredients present in red pepper and ginger. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, **402**, 259-267.

81. Vimala, S, Norhanom, AW and Yadav, M (1999) Anti-tumour promoter activity in Malaysian ginger rhizobia used in traditional medicine. *British Journal of cancer*, **80**, 110-116.

82. Chun, KS, Sohn, Y, Kim, HS, Kim, OH, Park, KK, Lee, J-M, Lee, J., Lee, JY, Moon, A, Lee, SS and Surh, YJ (1999) Anti-tumor promoting potential of

naturally occurring diarylheptanoids structurally related to curcumin. *Mutation Research*, **428**, 49-57.

83. Saruwatari, J, Nakagawa, K, Shindo, J, Nachi, S, Echizen, H and Ishizaki, T (2003) The in-vivo effects of sho-saiko-to, a traditional Chinese herbal medicine on two cytochrome P450 enzymes (1A2 and 3A) and xanthine oxidase in man. *Journal of Pharmacy and Pharmacology*, **55**, 1553-1559.

84. Shukla, Y. and Singh, M. (2006) Cancer preventive properties of ginger: A brief review. *Food Chem Toxicol*., **45**, 683-690.

85. Ihlaseh, S. M., de Oliveira, M. L., Teran, E., de Camargo, J. L. and Barbisan, L. F. (2006) Chemopreventive property of dietary ginger in rat urinary bladder chemical carcinogenesis. *World J Urol*, **24**, 591-596.

86. Manju, V. and Nalini, N. (2005) Chemopreventive efficacy of ginger, a naturally occurring anticarcinogen during the initiation, post-initiation stages of 1,2 dimethylhydrazine-induced colon cancer. *Clin Chim Acta*, **358**, 60-67.

87. Nagasawa, H., Watanabe, K. and Inatomi, H. (2002) Effects of bitter melon (Momordica charantia l.) or ginger rhizome (Zingiber offifinale rosc) on spontaneous mammary tumorigenesis in SHN mice. *Am J Chin Med*, **30**, 195-205.

88. Katiyar, S. K., Agarwal, R. and Mukhtar, H. (1996) Inhibition of tumor promotion in SENCAR mouse skin by ethanol extract of Zingiber officinale rhizome. *Cancer Res*, **56**, 1023-1030.

89. Park, K. K., Chun, K. S., Lee, J. M., Lee, S. S. and Surh, Y. J. (1998) Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice. *Cancer Lett*, **129**, 139-144.

90. Surh, Y. J., Park, K. K., Chun, K. S., Lee, L. J., Lee, E. and Lee, S. S. (1999) Anti-tumor-promoting activities of selected pungent phenolic substances present in ginger. *J Environ Pathol Toxicol Oncol*, **18**, 131-139.

91. Park, Y. J., Wen, J., Bang, S., Park, S. W. and Song, S. Y. (2006) [6]-Gingerol induces cell cycle arrest and cell death of mutant p53-expressing pancreatic cancer cells. *Yonsei Med J*, **47**, 688-697.

92. Chen, C.-Y., Liu, T.-Z., Liu, Y.-W., Tseng, W.-C., Liu, R.H., Lu, F.-J., Lin, Y.-S., Kuo, S.-H. and Chen, C.-H. (2007) 6-Shogaol (Alkanone from Ginger) Induces Apoptotic Cell Death of Human Hepatoma p53 Mutant Mahlavu Subline via an Oxidative Stress-Mediated Caspase-Dependent Mechanism. *J. Agric. Food Chem.,* **55**, 948-954.

93. Lee, E. and Surh, Y. J. (1998) Induction of apoptosis in HL-60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. *Cancer Lett*, **134**, 163-168.

94. Keum, Y. S., Kim, J., Lee, K. H., Park, K. K., Surh, Y. J., Lee, J. M., Lee, S. S., Yoon, J. H., Joo, S. Y., Cha, I. H. and Yook, J. I. (2002) Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. *Cancer Lett*, **177**, 41-47.

95. Bode, A. M., Ma, W. Y., Surh, Y. J. and Dong, Z. (2001) Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. *Cancer Res*, **61**, 850-853.

96. Wei, Q. Y., Ma, J. P., Cai, Y. J., Yang, L. and Liu, Z. L. (2005) Cytotoxic and apoptotic activities of diarylheptanoids and gingerol-related compounds from the rhizome of Chinese ginger. *J Ethnopharmacol*, **102**, 177-184.

97. Miyoshi, N., Nakamura, Y., Ueda, Y., Abe, M., Ozawa, Y., Uchida, K. and Osawa, T. (2003) Dietary ginger constituents, galanals A and B, are potent apoptosis inducers in Human T lymphoma Jurkat cells. *Cancer Lett*, **199**, 113-119.

98. Unnikrishnan, M. C. and Kuttan, R. (1988) Cytotoxicity of extracts of spices to cultured cells. *Nutr Cancer*, **11**, 251-257.

99. Kim, E. C., Min, J. K., Kim, T. Y., Lee, S. J., Yang, H. O., Han, S., Kim, Y. M. and Kwon, Y. G. (2005) [6]-Gingerol, a pungent ingredient of ginger, inhibits angiogenesis in vitro and in vivo. *Biochem Biophys Res Commun*, **335**, 300-308.

100. Mahady, G. B., Pendland, S. L., Yun, G. S., Lu, Z. Z. and Stoia, A. (2003) Ginger (Zingiber officinale Roscoe) and the gingerols inhibit the growth of Cag A+ strains of Helicobacter pylori. *Anticancer Res*, **23**, 3699-3702.

101. Kobayashi, M., Shoji, N. and Ohizumi, Y. (1987) Gingerol, a novel cardiotonic agent, activates the Ca2+-pumping ATPase in skeletal and cardiac sarcoplasmic reticulum. *Biochim Biophys Acta*, **903**, 96-102.

102. Suekawa, M., Ishige, A., Yuasa, K., Sudo, K., Aburada, M. and Hosoya, E. (1984) Pharmacological studies on ginger. I. Pharmacological actions of pungent constituents, (6)-gingerol and (6)-shogaol. *J Pharmacobiodyn*, **7**, 836-848.

103. Akhani, SP, Vishwakarma, SL and Goyal, RK (2004) Anti-diabetic activity of Zingiber officinale in streptozotocin-induced type I diabetic rats. *Journal of Pharmacy and Pharmacology*, **56**, 101-105.

104. Fuhrman, B, Rosenblat, M, Hayek, T, Coleman, R and Aviram, M (2000) Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice. *Journal of Nutrition*, **130**, 1124-1131.

105. Bhandari, U, Sharma, JN and Zafar, R (1998) The protective action of ethanolic ginger (Zingiber officinale) extract in cholesterol fed rabbits. *Journal of Ethnopharmacology*, **61**, 167-171.

106. Ojewole, J. A. (2006) Analgesic, antiinflammatory and hypoglycaemic effects of ethanol extract of Zingiber officinale (roscoe) rhizomes (zingiberaceae) in mice and rats. *Phytother Res*., 20 (9), 764-772.

107. Kato, A., Higuchi, Y., Goto, H., Kizu, H., Okamoto, T., Asano, N., Hollinshead, J., Nash, R. J. and Adachi, I. (2006) Inhibitory effects of Zingiber officinale Roscoe derived components on aldose reductase activity in vitro and in vivo. *J Agric Food Chem*, **54**, 6640-6644.

108. Abraham, S, Abraham, SK and Radhamany, G (1976) Mutagenic potential of the condiments ginger and turmeric. *Cytologia*, **41**, 591-595.

109. Mukhopadhyay, MJ and Mukherjee, A (2000) Clastogenic effect of ginger rhizome in mice. *Phytotherapy Research*, **14**, 555-557.

110. Wilkinson, J. M. (2000) Effect of ginger tea on the fetal development of Sprague-Dawley rats. *Reproductive Toxicology*, **14**, 507-512.

111. Weidner, M. S. and Sigwart, K. (2001) Investigation of the teratogenic potential of a zingiber officinale extract in the rat. *Reprod Toxicol*, **15**, 75-80.

112. Weidner, M. S. and Sigwart, K. (2000) The safety of a ginger extract in the rat. *Journal of Ethnopharmacology*, **73**, 513-520.

113. Nakazawa, T and Ohsawa, K (2002) Metabolism of [6]-gingerol in rats. *Life Sciences*, **70**, 2165-2175.

114. Pfeiffer, E., Heuschmid, F. F., Kranz, S. and Metzler, M. (2006) Microsomal hydroxylation and glucuronidation of [6]-gingerol. *J Agric Food Chem*, **54**, 8769-8774.

115. Surh, Y. J. and Lee, S. S. (1992) Enzymatic Reduction of shogaol:a novel biotransformation pathway for the alpha,beta-unsaturated ketone system. *Biochemistry International*, **27**, 178-187.

116. Ding, G. H., Naora, K., Hayashibara, M., Katagiri, Y., Kano, Y. and Iwamoto, K. (1991) Pharmacokinetics of [6]-gingerol after intravenous administration in rats. *Chem Pharm Bull (Tokyo)*, **39**, 1612-1614.

117. Bray, F I and Ferlay, J (2003) Global burden of cancer. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 11-20.

118. Oppenheimer, Steven B. (1984) *Cancer: A biological and clinical introduction*. Jones and Bartlett Publishers, Inc., Northridge.

119. Cancer Facts and Figures. (2006). American Cancer society, Atlanta.

120. Ames, B. N., Gold, L. S. and Willett, W. C. (1995) The causes and prevention of cancer. *Proc Natl Acad Sci U S A*, **92**, 5258-5265.

121. Prasad, K. N., Cole, W. and Hovland, P. (1998) Cancer prevention studies: past, present, and future directions. *Nutrition*, **14**, 197-210.

122. Bray, F I and Ferlay, J (2003) Globocan 2000. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 238.

123. Boffetta, P (2003) Tobacco. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 22-28.

124. Boffetta, P (2003) Alcohol drinking. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 29-32.

125. Boffetta, P (2003) Occupational cancer. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 33.

126. Boffetta, P (2003) Environmental pollution. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 39-42.

127. Hainaut, P (2003) Food contaminants. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 43-47.

128. Rice, J (2003) Medicinal drugs. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 48-50.

129. Riboli, E (2003) Diet and nutrition. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 62-67.

130. Rice, J (2003) Immunosuppression. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 68-70.

131. Kaaks, R (2003) Reproductive factors and hormones. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 76-82.

132. Goldgar, D (2003) Genetic susceptibility. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 71-75.

133. De Flora, S. and Ferguson, L. R. (2005) Overview of mechanisms of cancer chemopreventive agents. *Mutat Res*, **591**, 8-15.

134. Ferguson, L. R. (1994) Antimutagens as cancer chemopreventive agents in the diet. *Mutat Res*, **307**, 395-410.

135. Rock, C. L., Lampe, J. W. and Patterson, R. E. (2000) Nutrition, genetics, and risks of cancer. *Annu Rev Public Health*, **21**, 47-64.

136. Martin, Seamus J. (1997) Apoptosis and Cancer: An overview. In Martin, S.J. (ed.), *Apoptosis and Cancer*. Karger Landes Systems, Basel, Switzerland, pp. 1-8.

137. Macdonald, F, Ford, CHJ and Casson, AG (2004) *Molecular biology of cancer*. BIOS scientific publisher, London and New York.

138. Tessema, M., Lehmann, U. and Kreipe, H. (2004) Cell cycle and no end. *Virchows Arch*, **444**, 313-323.

139. Heath, John K. (2001) *Principles of cell proliferation*. Blackwell Science, Birmingham.

140. Stewart, BW and Yamasaki, H (2003) Multistage carcinogenesis. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 84-88.

141. Yamasaki, H (2003) Cell-cell communication. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 84-88.

142. Stewart, BW (2003) Apoptosis. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 113-118.

143. Hainaut, P (2003) Cell cycle. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 104-108.

144. Eccles, S (2003) Invasion and metastasis. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 119-126.

145. Barbin, A (2003) Carcinogen activation and DNA repair. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 89-95.

146. Hainaut, P (2003) Oncogenes and tumour suppressor genes. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 96-103.

147. Pierce, BG (1998) The pathology of cancer. In Pierce, B. (ed.), *The biological basis of cancer*. Cambridge university press, Cambridge, UK, pp. 14-49.

148. Loeb, L. A., Loeb, K. R. and Anderson, J. P. (2003) Multiple mutations and cancer. *Proc Natl Acad Sci U S A*, **100**, 776-781.

149. Brown, J. M. and Wouters, B. G. (1999) Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res*, **59**, 1391-1399.

150. Vainio (2003) Chemoprevention. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 151-155.

151. Chiarugi, V., Cinelli, M., Magnelli, L. and Dello, S. P. (2002) Apoptosis: molecular regulation of cell death and hematologic malignancies. *Mol Biotechnol*, **20**, 305-314.

152. Broker, L. E., Kruyt, F. A. and Giaccone, G. (2005) Cell death independent of caspases: a review. *Clin Cancer Res*, **11**, 3155-3162.

153. Delhalle, S., Duvoix, A., Schnekenburger, M., Morceau, F., Dicato, M. and Diederich, M. (2003) An introduction to the molecular mechanisms of apoptosis. *Ann N Y Acad Sci*, **1010**, 1-8.

154. Dash, Phil (2005) Apoptosis. http://www.sgul.ac.uk/depts/immunology/~dash/.

155. Gewies, Andreas (2003) ApoReview: Introduction to Apoptosis. www.celldeath.de.

156. Toft, Neil J. and Arends, Mark J. (1997) Apoptosis and Necrosis in Tumors. In Martin, S.J. (ed.), *Apoptosis and Cancer*. Karger Landes Systems, Basel, Switzerland, pp. 25-44.

157. Ioannou, Y. A. and Chen, F. W. (1996) Quantitation of DNA fragmentation in apoptosis. *Nucleic Acids Res*, **24**, 992-993.

158. Martin, Seamus J. (1997) Apoptosis: An Introduction. In Martin, S.J. (ed.), *Apoptosis and Cancer*. Karger Landes Systems, Basel, Switzerland, pp. 9-24.

159. Mates, J. M. and Sanchez-Jimenez, F. M. (2000) Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int J Biochem Cell Biol*, **32**, 157-170.

160. Perl, M., Chung, C. S. and Ayala, A. (2005) Apoptosis. *Crit Care Med*, **33**, S526-S529.

161. Bras, M., Queenan, B. and Susin, S. A. (2005) Programmed cell death via mitochondria: different modes of dying. *Biochemistry (Mosc)*, **70**, 231-239.

162. Watson, R. W. and Fitzpatrick, J. M. (2005) Targeting apoptosis in prostate cancer: focus on caspases and inhibitors of apoptosis proteins. *BJU Int*, **96 Suppl 2**, 30-34.

163. Schlegel, R. A., Callahan, M. K. and Williamson, P. (2000) The central role of phosphatidylserine in the phagocytosis of apoptotic thymocytes. *Ann N Y Acad Sci*, **926**, 217-225.

164. Williamson, P. and Schlegel, R. A. (2002) Transbilayer phospholipid movement and the clearance of apoptotic cells. *Biochim Biophys Acta*, **1585**, 53-63.

165. Kagan, V. E., Fabisiak, J. P., Shvedova, A. A., Tyurina, Y. Y., Tyurin, V. A., Schor, N. F. and Kawai, K. (2000) Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Lett*, **477**, 1-7.

166. Van Cruchten, S. and Van Den Broeck, W. (2002) Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Histol Embryol*, **31**, 214-23.

167. Mallat, Z. and Tedgui, A. (2000) Apoptosis in the vasculature: mechanisms and functional importance. *Br J Pharmacol*, **130**, 947-962.

168. Apoptosis. (2005). Promega.

169. Ghobrial, I. M., Witzig, T. E. and Adjei, A. A. (2005) Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin*, **55**, 178-194.

170. Hussein, M. R., Haemel, A. K. and Wood, G. S. (2003) Apoptosis and melanoma: molecular mechanisms. *J Pathol*, **199**, 275-288.

171. Danial, N. N. and Korsmeyer, S. J. (2004) Cell death: critical control points. *Cell*, **116**, 205-219.

172. Fraser, A. and Evan, G. (1996) A license to kill. *Cell*, **85**, 781-784.

173. Osborne, B. A. (1995) Induction of genes during apoptosis: examples from the immune system. *Semin Cancer Biol*, **6**, 27-33.

174. Abe, K., Kurakin, A., Mohseni-Maybodi, M., Kay, B. and Khosravi-Far, R. (2000) The complexity of TNF-related apoptosis-inducing ligand. *Ann N Y Acad Sci*, **926**, 52-63.

175. MacFarlane, M. and Williams, A. C. (2004) Apoptosis and disease: a life or death decision. *EMBO Rep*, **5**, 674-678.

176. Rowan, S. and Fisher, D. E. (1997) Mechanisms of apoptotic cell death. *Leukemia*, **11**, 457-465.

177. Bursch, W., Ellinger, A., Gerner, C., Frohwein, U. and Schulte-Hermann, R. (2000) Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? *Ann N Y Acad Sci*, **926**, 1-12.

178. Lin, K. T., Xue, J. Y., Nomen, M., Spur, B. and Wong, P. Y. (1995) Peroxynitrite-induced apoptosis in HL-60 cells. *J Biol Chem*, **270**, 16487-16490.

179. Simon, H. U., Haj-Yehia, A. and Levi-Schaffer, F. (2000) Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, **5**, 415-418.

180. Stvrtinova, V, Jakubovsky, J and Hulin, I (1995) *Pathophysiology principles of disease*. Academic electronic press; http://www.savba.sk/logos/books/scientific/Inffever.html.

181. Tipp, A (1979) *Basic pathophysiological mechanisms of inflammation*. Mc Graw-Hill book company, Lamoni, Iowa, USA.

182. Ager, A. (2003) Inflammation: Border crossings. *Nature*, **421**, 703-705.

183. Finkel, T. and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature*, **408**, 239-247.

184. Finkel, T. (2003) Oxidant signals and oxidative stress. *Curr Opin Cell Biol*, **15**, 247-254.

185. Splettstoesser, W. D. and Schuff-Werner, P. (2002) Oxidative stress in phagocytes--"the enemy within". *Microsc Res Tech*, **57**, 441-455.

186. D'Angio, C. T. and Finkelstein, J. N. (2000) Oxygen regulation of gene expression: a study in opposites. *Mol Genet Metab*, **71**, 371-380.

187. Nelms, Keats (2000) Cytokine Signal transduction. In Balkwill, F. (ed.), *The cytokine network*. Oxford University press, London, UK, pp. 20-48.

188. Brennan, Fionula M. and Feldmann, Marc (2000) Cytokine Networks. In Balkwill, F. (ed.), *The cytokine network*. Oxford University press, London, UK, pp. 49-70.

189. Fernandez-Botran, Rafael and Vetvicka, Vaclav (2001) *Methods in cellular imunology*. CRC Press.

190. Tracey, K. J. (2002) The inflammatory reflex. *Nature*, **420**, 853-859.

191. Hazuda, D. J., Strickler, J., Kueppers, F., Simon, P. L. and Young, P. R. (1990) Processing of precursor interleukin 1 beta and inflammatory disease. *J Biol Chem*, **265**, 6318-6322.

192. Hinson, R. M., Williams, J. A. and Shacter, E. (1996) Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: possible role of cyclooxygenase-2. *Proc Natl Acad Sci U S A*, **93**, 4885-4890.

193. Hirano, Toshio (1998) Interleukin-6. In Thomson, A.W. (ed.), *The Cytokine Handbook*. Academic Press Limited, Denver, Colorado, pp. 197-228.

194. Dieter, P (1999) The generation of free arachidonic acid. In Furstenberger, G. (ed.), *Prostaglandins, Leukotrienes and essential fatty acids*. Wiley-VCH, Heidelberg, Germany, pp. 47-64.

195. Marks, F and Furstenberger, G (1999) Eicosanoids and cancer. In Furstenberger, G. (ed.), *Prostaglandins, Leukotrienes and essential fatty acids*. Wiley-VCH, Heidelberg, Germany, pp. 303-330.

196. Claria, J. and Romano, M. (2005) Pharmacological intervention of cyclooxygenase-2 and 5-lipoxygenase pathways. Impact on inflammation and cancer. *Curr Pharm Des*, **11**, 3431-3447.

197. Chandrasekharan, N. V., Dai, H., Roos, K. L., Evanson, N. K., Tomsik, J., Elton, T. S. and Simmons, D. L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A*, **99**, 13926-13931.

198. Hong, S. H., Avis, I., Vos, M. D., Martinez, A., Treston, A. M. and Mulshine, J. L. (1999) Relationship of arachidonic acid metabolizing enzyme expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors. *Cancer Res*, **59**, 2223-2228.

199. Kuhn, H (1999) Lipoxygenases. In Furstenberger, G. (ed.), *Prostaglandins, Leukotrienes and essential fatty acids*. Wiley-VCH, Heidelberg, Germany, pp. 109-142.

200. Samuelsson, B. and Funk, C. D. (1989) Enzymes involved in the biosynthesis of leukotriene B4. *J Biol Chem*, **264**, 19469-19472.

201. Cordon-Cardo, C. and Prives, C. (1999) At the crossroads of inflammation and tumorigenesis. *J Exp Med*, **190**, 1367-1370.

202. Lu, H., Ouyang, W. and Huang, C. (2006) Inflammation, a Key Event in Cancer Development. *Mol Cancer Res*., **4**, 221-233.

203. Volk, T., Gerst, J., Faust-Belbe, G., Stroehmann, A. and Kox, W. J. (1999) Monocyte stimulation by reactive oxygen species: role of superoxide and intracellular Ca2+. *Inflamm Res*, **48**, 544-549.

204. Furstenberger, G., Krieg, P., Muller-Decker, K. and Habenicht, A. J. (2006) What are cyclooxygenases and lipoxygenases doing in the driver's seat of carcinogenesis? *Int J Cancer*, **119**, 2247-2254.

205. Moss, S. F. and Blaser, M. J. (2005) Mechanisms of disease: Inflammation and the origins of cancer. *Nat Clin Pract Oncol*, **2**, 90-97.

206. Schottenfeld, D. and Beebe-Dimmer, J. (2006) Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. *CA Cancer J Clin*, **56**, 69-83.

207. Nam, J.H. and Murthy, S (2004) Chronic inflammation and cancer in various organ systems. In Nakada, M.T. (ed.), *Cancer and inflammation*. Birkhauser verlag, Basel, Switzerland, pp. 1-20.

208. Hussain, S. P., Hofseth, L. J. and Harris, C. C. (2003) Radical causes of cancer. *Nat Rev Cancer*, **3**, 276-285.

209. Thun, M.J., Henley, S.J. and Gansler, T (2004) Inflammation and cancer: an epidemiological perspective. In Gordon, S. (ed.), *Cancer and Inflammation*. John Wiley and Sons, Ltd, Chichester, UK, pp. 6-28.

210. Balkwill, F. and Mantovani, A. (2001) Inflammation and cancer: back to Virchow? *Lancet*, **357**, 539-545.

211. Lotze, M.T. and Herberman, R.B. (2004) Cancer as a chronic inflammatory disease: role of immunotherapy. In Nakada, M.T. (ed.), *Cancer and inflammation*. Birkhauser verlag, Basel, Switzerland, pp. 21-52.

212. Coussens, L. M. and Werb, Z. (2002) Inflammation and cancer. *Nature*, **420**, 860-867.

213. Vakkila, J. and Lotze, M. T. (2004) Inflammation and necrosis promote tumour growth. *Nat Rev Immunol*, **4**, 641-648.

214. Romano, M., Catalano, A., Nutini, M., D'Urbano, E., Crescenzi, C., Claria, J., Libner, R., Davi, G. and Procopio, A. (2001) 5-lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. *Faseb J*, **15**, 2326-2336.

215. Anderson, K. M., Seed, T., Vos, M., Mulshine, J., Meng, J., Alrefai, W., Ou, D. and Harris, J. E. (1998) 5-Lipoxygenase inhibitors reduce PC-3 cell proliferation and initiate nonnecrotic cell death. *Prostate*, **37**, 161-173.

216. Avis, I. M., Jett, M., Boyle, T., Vos, M. D., Moody, T., Treston, A. M., Martinez, A. and Mulshine, J. L. (1996) Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. *J Clin Invest*, **97**, 806-813.

217. Dannenberg, A. J., Lippman, S. M., Mann, J. R., Subbaramaiah, K. and DuBois, R. N. (2005) Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. *J Clin Oncol*, **23**, 254-266.

218. Li, N., Sood, S., Wang, S., Fang, M., Wang, P., Sun, Z., Yang, C. S. and Chen, X. (2005) Overexpression of 5-lipoxygenase and cyclooxygenase 2 in hamster and human oral cancer and chemopreventive effects of zileuton and celecoxib. *Clin Cancer Res*, **11**, 2089-2096.

219. Mazhar, D., Gillmore, R. and Waxman, J. (2005) COX and cancer. *Qjm*, **98**, 711-718.

220. Qiao, L., Kozoni, V., Tsioulias, G. J., Koutsos, M. I., Hanif, R., Shiff, S. J. and Rigas, B. (1995) Selected eicosanoids increase the proliferation rate of human colon carcinoma cell lines and mouse colonocytes in vivo. *Biochim Biophys Acta*, **1258**, 215-223.

221. Shamma, A., Yamamoto, H., Doki, Y., Okami, J., Kondo, M., Fujiwara, Y., Yano, M., Inoue, M., Matsuura, N., Shiozaki, H. and Monden, M. (2000) Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. *Clin Cancer Res*, **6**, 1229-1238.

222. Gupta, S., Srivastava, M., Ahmad, N., Sakamoto, K., Bostwick, D. G. and Mukhtar, H. (2001) Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer*, **91**, 737-743.

223. Hennig, R., Grippo, P., Ding, X. Z., Rao, S. M., Buchler, M. W., Friess, H., Talamonti, M. S., Bell, R. H. and Adrian, T. E. (2005) 5-Lipoxygenase, a marker for early pancreatic intraepithelial neoplastic lesions. *Cancer Res*, **65**, 6011-6016.

224. Yoshimura, R., Matsuyama, M., Tsuchida, K., Kawahito, Y., Sano, H. and Nakatani, T. (2003) Expression of lipoxygenase in human bladder carcinoma and growth inhibition by its inhibitors. *J Urol*, **170**, 1994-1999.

225. Yoshimura, R., Matsuyama, M., Mitsuhashi, M., Takemoto, Y., Tsuchida, K., Kawahito, Y., Sano, H. and Nakatani, T. (2004) Relationship between lipoxygenase and human testicular cancer. *Int J Mol Med*, **13**, 389-393.

226. Ohd, J. F., Nielsen, C. K., Campbell, J., Landberg, G., Lofberg, H. and Sjolander, A. (2003) Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. *Gastroenterology*, **124**, 57-70.

227. Buskens, C. J., Ristimaki, A., Offerhaus, G. J., Richel, D. J. and van Lanschot, J. J. (2003) Role of cyclooxygenase-2 in the development and treatment of oesophageal adenocarcinoma. *Scand J Gastroenterol Suppl*, 87-93.

228. Buskens, C. J., Sivula, A., van Rees, B. P., Haglund, C., Offerhaus, G. J., van Lanschot, J. J. and Ristimaki, A. (2003) Comparison of cyclooxygenase 2 expression in adenocarcinomas of the gastric cardia and distal oesophagus. *Gut*, **52**, 1678-1683.

229. Buskens, C. J., Van Rees, B. P., Sivula, A., Reitsma, J. B., Haglund, C., Bosma, P. J., Offerhaus, G. J., Van Lanschot, J. J. and Ristimaki, A. (2002) Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. *Gastroenterology*, **122**, 1800-1807.

230. Denkert, C., Winzer, K. J. and Hauptmann, S. (2004) Prognostic impact of cyclooxygenase-2 in breast cancer. *Clin Breast Cancer*, **4**, 428-433.

231. Gao, X., Grignon, D. J., Chbihi, T., Zacharek, A., Chen, Y. Q., Sakr, W., Porter, A. T., Crissman, J. D., Pontes, J. E., Powell, I. J. and et al. (1995) Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. *Urology*, **46**, 227-237.

232. Gupta, S., Srivastava, M., Ahmad, N., Bostwick, D. G. and Mukhtar, H. (2000) Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. *Prostate*, **42**, 73-78.

233. Hennig, R., Ding, X. Z., Tong, W. G., Schneider, M. B., Standop, J., Friess, H., Buchler, M. W., Pour, P. M. and Adrian, T. E. (2002) 5-Lipoxygenase and leukotriene B(4) receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue. *Am J Pathol*, **161**, 421-428.

234. Kawamori, T., Uchiya, N., Sugimura, T. and Wakabayashi, K. (2003) Enhancement of colon carcinogenesis by prostaglandin E2 administration. *Carcinogenesis*, **24**, 985-990.

235. Saukkonen, K., Rintahaka, J., Sivula, A., Buskens, C. J., Van Rees, B. P., Rio, M. C., Haglund, C., Van Lanschot, J. J., Offerhaus, G. J. and Ristimaki, A. (2003) Cyclooxygenase-2 and gastric carcinogenesis. *Apmis*, **111**, 915-925.

236. Sivula, A., Buskens, C. J., van Rees, B. P., Haglund, C., Offerhaus, G. J., van Lanschot, J. J. and Ristimaki, A. (2005) Prognostic role of cyclooxygenase-2 in neoadjuvant-treated patients with squamous cell carcinoma of the esophagus. *Int J Cancer*, **116**, 903-908.

237. Wang, D., Buchanan, F. G., Wang, H., Dey, S. K. and DuBois, R. N. (2005) Prostaglandin E2 enhances intestinal adenoma growth via activation of the Ras-mitogen-activated protein kinase cascade. *Cancer Res*, **65**, 1822-1829.

238. Chen, X., Wang, S., Wu, N., Sood, S., Wang, P., Jin, Z., Beer, D. G., Giordano, T. J., Lin, Y., Shih, W. C., Lubet, R. A. and Yang, C. S. (2004) Overexpression of 5-lipoxygenase in rat and human esophageal adenocarcinoma and inhibitory effects of zileuton and celecoxib on carcinogenesis. *Clin Cancer Res*, **10**, 6703-6709.

239. Sood, S., Shiff, S. J., Yang, C. S. and Chen, X. (2005) Selection of topically applied non-steroidal anti-inflammatory drugs for oral cancer chemoprevention. *Oral Oncol*, **41**, 562-567.

240. Chen, X., Sood, S., Yang, C. S., Li, N. and Sun, Z. (2006) Five-lipoxygenase pathway of arachidonic acid metabolism in carcinogenesis and cancer chemoprevention. *Current Cancer Dug Targets*, **6**, 519-532.

241. Sun, Z., Sood, S., Li, N., Ramji, D., Yang, P., Newman, R. A., Yang, C. S. and Chen, X. (2006) Involvement of the 5-lipoxygenase/leukotriene A4 hydrolase pathway in 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch, and inhibition of carcinogenesis by its inhibitors. *Carcinogenesis*, **27**, 1902-1908.

242. Tsao, A. S., Kim, E. S. and Hong, W. K. (2004) Chemoprevention of cancer. *CA Cancer J Clin*, **54**, 150-180.

243. Mann, J. R., Backlund, M. G. and DuBois, R. N. (2005) Mechanisms of disease: Inflammatory mediators and cancer prevention. *Nat Clin Pract Oncol*, **2**, 202-210.

244. Edderkaoui, M., Hong, P., Vaquero, E. C., Lee, J. K., Fischer, L., Friess, H., Buchler, M. W., Lerch, M. M., Pandol, S. J. and Gukovskaya, A. S. (2005) Extracellular matrix stimulates reactive oxygen species production and increases pancreatic cancer cell survival through 5-lipoxygenase and NADPH oxidase. *Am J Physiol Gastrointest Liver Physiol*, **289**, G1137-G1147.

245. Charlier, C. and Michaux, C. (2003) Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. *Eur J Med Chem*, **38**, 645-659.

246. Gupta, S., Adhami, V. M., Subbarayan, M., MacLennan, G. T., Lewin, J. S., Hafeli, U. O., Fu, P. and Mukhtar, H. (2004) Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. *Cancer Res*, **64**, 3334-3343.

247. Lev-Ari, S., Strier, L., Kazanov, D., Madar-Shapiro, L., Dvory-Sobol, H., Pinchuk, I., Marian, B., Lichtenberg, D. and Arber, N. (2005) Celecoxib and curcumin synergistically inhibit the growth of colorectal cancer cells. *Clin Cancer Res*, **11**, 6738-6744.

248. Reddy, B. S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K. and Rao, C. V. (2000) Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res*, **60**, 293-297.

249. Tong, W. G., Ding, X. Z., Witt, R. C. and Adrian, T. E. (2002) Lipoxygenase inhibitors attenuate growth of human pancreatic cancer xenografts and induce apoptosis through the mitochondrial pathway. *Mol Cancer Ther*, **1**, 929-935.

250. Gann, P. H., Manson, J. E., Glynn, R. J., Buring, J. E. and Hennekens, C. H. (1993) Low-dose aspirin and incidence of colorectal tumors in a randomized trial. *J Natl Cancer Inst*, **85**, 1220-1224.

251. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R. and Offerhaus, G. J. (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med*, **328**, 1313-1316.

252. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K. and Levin, B. (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med*, **342**, 1946-1952.

253. Baron, J. A., Cole, B. F., Sandler, R. S., Haile, R. W., Ahnen, D., Bresalier, R., McKeown-Eyssen, G., Summers, R. W., Rothstein, R., Burke, C. A., Snover, D. C., Church, T. R., Allen, J. I., Beach, M., Beck, G. J., Bond, J. H., Byers, T., Greenberg, E. R., Mandel, J. S., Marcon, N., Mott, L. A., Pearson, L., Saibil, F. and van Stolk, R. U. (2003) A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med*, **348**, 891-899.

254. Sandler, R. S., Halabi, S., Baron, J. A., Budinger, S., Paskett, E., Keresztes, R., Petrelli, N., Pipas, J. M., Karp, D. D., Loprinzi, C. L., Steinbach, G. and Schilsky, R. (2003) A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med*, **348**, 883-890.

255. Pollard, J. W. (2004) Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*, **4**, 71-78.

256. Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J. and Beach, D. H. (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J Exp Med*, **190**, 1375-1382.

257. Karin, M. (2005) Inflammation and cancer: the long reach of Ras. *Nat Med*, **11**, 20-21.

258. Sparmann, A. and Bar-Sagi, D. (2005) Ras oncogene and inflammation: partners in crime. *Cell Cycle*, **4**, 735-736.

259. Sparmann, A. and Bar-Sagi, D. (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell*, **6**, 447-458.

260. Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T. and Akira, S. (1993) Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U S A*, **90**, 10193-10197.

261. Suematsu, S., Matsusaka, T., Matsuda, T., Hirano, T. and Kishimoto, T. (1993) Interleukin-6 in myeloma/plasmacytoma. *Int Rev Exp Pathol*, **34 Pt A**, 91-98.

262. Jaiswal, M., LaRusso, N. F., Burgart, L. J. and Gores, G. J. (2000) Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res*, **60**, 184-190.

263. Jaiswal, M., LaRusso, N. F., Shapiro, R. A., Billiar, T. R. and Gores, G. J. (2001) Nitric oxide-mediated inhibition of DNA repair potentiates oxidative DNA damage in cholangiocytes. *Gastroenterology*, **120**, 190-199.

264. Suganuma, M., Okabe, S., Kurusu, M., Iida, N., Ohshima, S., Saeki, Y., Kishimoto, T. and Fujiki, H. (2002) Discrete roles of cytokines, TNF-alpha, IL-1, IL-6 in tumor promotion and cell transformation. *Int J Oncol*, **20**, 131-136.

265. Weitzman, S. A. and Gordon, L. I. (1990) Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood*, **76**, 655-663.

266. Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A*, **90**, 7915-7922.

267. Herrera, B., Alvarez, A. M., Sanchez, A., Fernandez, M., Roncero, C., Benito, M. and Fabregat, I. (2001) Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transforming growth factor (beta) in fetal hepatocytes. *Faseb J*, **15**, 741-751.

268. Gorman, A., McGowan, A. and Cotter, T. G. (1997) Role of peroxide and superoxide anion during tumour cell apoptosis. *FEBS Lett*, **404**, 27-33.

269. Cai, J. and Jones, D. P. (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J Biol Chem*, **273**, 11401-11404.

270. Cancer: how not to go there. The secret to cancer prevention may be in the gym. Or in the spice rack. Let's hope researchers find it soon. (2004) *Harvard Health Letters*, **29**, 4.

271. Burton, A (2003) Chemoprevention: Eat ginger rub on pomegranate. *The Lancet oncology*, **4**, 715.

272. Bode, AM and Dong, Z (2004) Targeting signal transduction pathways by chemopreventive agents. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **555**, 33-51.

273. Zingiber officinale (ginger). Monograph. (2003) *Alternative Medicinal Review*, **8**, 331-335.

274. Afzal, M, Al-Hadidi, D, Menon, M, Pasek, J and Dhami, MSI (2001) Ginger:An ethnomedical, chemical and pharmacological review. *Drug metabolism and drug interactions*, **18**, 159-190.

275. Tonelli, G., Thibault, L. and Ringler, I. (1965) A bio-assay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids. *Endocrinology*, **77**, 625-634.

276. Boris, A. and Hurley, J. F. (1977) Assessment of topical anti-inflammatory activity in rats with cantharidin-induced inflammation. *J Invest Dermatol*, **68**, 161-164.

277. Glenn, E. M., Bowman, B. J. and Rohloff, N. A. (1978) Simple laboratory procedures for the evaluation of topically-active anti-inflammatory drugs. *Agents Actions*, **8**, 497-503.

278. Huang, M. T., Smart, R. C., Wong, C. Q. and Conney, A. H. (1988) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res*, **48**, 5941-5946.

279. Katiyar, S. K., Rupp, C. O., Korman, N. J., Agarwal, R. and Mukhtar, H. (1995) Inhibition of 12-O-tetradecanoylphorbol-13-acetate and other skin tumor-promoter-caused induction of epidermal interleukin-1 alpha mRNA and protein expression in SENCAR mice by green tea polyphenols. *J Invest Dermatol*, **105**, 394-398.

280. Na, H. K., Mossanda, K. S., Lee, J. Y. and Surh, Y. J. (2004) Inhibition of phorbol ester-induced COX-2 expression by some edible African plants. *Biofactors*, **21**, 149-153.

281. Oberyszyn, T. M., Sabourin, C. L., Bijur, G. N., Oberyszyn, A. S., Boros, L. G. and Robertson, F. M. (1993) Interleukin-1 alpha gene expression and localization of interleukin-1 alpha protein during tumor promotion. *Mol Carcinog*, **7**, 238-248.

282. Suh, D. H., Youn, J. I. and Eun, H. C. (2001) Effects of 12-O-tetradecanoyl-phorbol-13-acetate [corrected] and sodium lauryl sulfate on the production and expression of cytokines and proto-oncogenes in photoaged and intrinsically aged human keratinocytes. *J Invest Dermatol*, **117**, 1225-1233.

283. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J. and Thun, M. J. (2007) Cancer statistics, 2007. *CA Cancer J Clin*, **57**, 43-66.

284. Pienta, K. J. and Smith, D. C. (2005) Advances in prostate cancer chemotherapy: a new era begins. *CA Cancer J Clin*, **55**, 300-318.

285. Ghosh, J. and Myers, C. E. (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem Biophys Res Commun*, **235**, 418-423.

286. Denis, LJ, Griffith, K and von Eschenbach, AC (2003) Prostate cancer. In Kleihues, P. (ed.), *World cancer report*. IARC press, Lyon, France, pp. 208-211.

287. Bruckheimer, E. M. and Kyprianou, N. (2000) Apoptosis in prostate carcinogenesis. A growth regulator and a therapeutic target. *Cell Tissue Res*, **301**, 153-162.

288. Feldman, B. J. and Feldman, D. (2001) The development of androgen-independent prostate cancer. *Nat Rev Cancer*, **1**, 34-45.

289. Canby-Hagino, E. D. and Thompson, I. M. (2005) Mechanisms of disease: Prostate cancer--a model for cancer chemoprevention in clinical practice. *Nat Clin Pract Oncol*, **2**, 255-261.

290. Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K. and Sandberg, A. A. (1980) The LNCaP cell line--a new model for studies on human prostatic carcinoma. *Prog Clin Biol Res*, **37**, 115-132.

291. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. and Jones, L. W. (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol*, **17**, 16-23.

292. Heisler, L. E., Evangelou, A., Lew, A. M., Trachtenberg, J., Elsholtz, H. P. and Brown, T. J. (1997) Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol Cell Endocrinol*, **126**, 59-73.

293. Ewing, C. M., Ru, N., Morton, R. A., Robinson, J. C., Wheelock, M. J., Johnson, K. R., Barrett, J. C. and Isaacs, W. B. (1995) Chromosome 5 suppresses tumorigenicity of PC3 prostate cancer cells: correlation with re-expression of alpha-catenin and restoration of E-cadherin function. *Cancer Res*, **55**, 4813-4817.

294. Veldscholte, J., Berrevoets, C. A. and Mulder, E. (1994) Studies on the human prostatic cancer cell line LNCaP. *J Steroid Biochem Mol Biol*, **49**, 341-346.

295. Veldscholte, J., Berrevoets, C. A., Ris-Stalpers, C., Kuiper, G. G., Jenster, G., Trapman, J., Brinkmann, A. O. and Mulder, E. (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J Steroid Biochem Mol Biol*, **41**, 665-669.

296. van Steenbrugge, G. J., van Uffelen, C. J., Bolt, J. and Schroder, F. H. (1991) The human prostatic cancer cell line LNCaP and its derived sublines: an in vitro model for the study of androgen sensitivity. *J Steroid Biochem Mol Biol*, **40**, 207-214.

297. Schuurmans, A. L., Bolt, J., Veldscholte, J. and Mulder, E. (1991) Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. *J Steroid Biochem Mol Biol*, **40**, 193-197.

298. Culig, Z., Klocker, H., Bartsch, G. and Hobisch, A. (2002) Androgen receptors in prostate cancer. *Endocr Relat Cancer*, **9**, 155-170.

299. Flaig, T. W., Su, L. J., Harrison, G., Agarwal, R. and Glode, L. M. (2007) Silibinin synergizes with mitoxantrone to inhibit cell growth and induce apoptosis in human prostate cancer cells. *Int J Cancer*, **120**, 2028-2033.

300. Shannan, B., Seifert, M., Boothman, D. A., Tilgen, W. and Reichrath, J. (2007) Clusterin over-expression modulates proapoptotic and antiproliferative effects of 1,25(OH)(2)D(3) in prostate cancer cells in vitro. *J Steroid Biochem Mol Biol*., **103**, 721-725.

301. Pinto, J. T., Sinha, R., Papp, K., Facompre, N. D., Desai, D. and El-Bayoumy, K. (2007) Differential effects of naturally occurring and synthetic organoselenium compounds on biomarkers in androgen responsive and androgen independent human prostate carcinoma cells. *Int J Cancer*, **120**, 1410-1417.

302. He, XG, Bernart, MW, Lian, LZ and Lin, LZ (1998) High-performance liquid chromatography electrospray mass spectrometric analysis of pungent constituents of ginger. *Journal of Chromatography A*, **796**, 327-334.

303. Kim, J. H., Xu, C., Keum, Y. S., Reddy, B., Conney, A. and Kong, A. N. (2006) Inhibition of EGFR signaling in human prostate cancer PC-3 cells by combination treatment with beta-phenylethyl isothiocyanate and curcumin. *Carcinogenesis*, **27**, 475-482.

304. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65**, 55-63.

305. Zheng, X., Chang, R. L., Cui, X. X., Avila, G. E., Lee, S., Lu, Y. P., Lou, Y. R., Shih, W. J., Lin, Y., Reuhl, K., Newmark, H., Rabson, A. and Conney, A. H. (2004) Inhibitory effect of 12-O-tetradecanoylphorbol-13-acetate alone or in combination with all-trans-retinoic acid on the growth of LNCaP prostate tumors in immunodeficient mice. *Cancer Res*, **64**, 1811-1820.

306. Crissman, H. A. and Steinkamp, J. A. (1973) Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J Cell Biol*, **59**, 766-771.

307. Krishan, A. (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J Cell Biol*, **66**, 188-193.

308. van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B. and Reutelingsperger, C. P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*, **31**, 1-9.

309. van Engeland, M., Ramaekers, F. C., Schutte, B. and Reutelingsperger, C. P. (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry*, **24**, 131-139.

310. Ashush, H., Rozenszajn, L. A., Blass, M., Barda-Saad, M., Azimov, D., Radnay, J., Zipori, D. and Rosenschein, U. (2000) Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. *Cancer Res*, **60**, 1014-1020.

311. Hou, Z., Sang, S., You, H., Lee, M. J., Hong, J., Chin, K. V. and Yang, C. S. (2005) Mechanism of action of (-)-epigallocatechin-3-gallate: auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. *Cancer Res*, **65**, 8049-8056.

312. Chen, C. H., Chern, C. L., Lin, C. C., Lu, F. J., Shih, M. K., Hsieh, P. Y. and Liu, T. Z. (2003) Involvement of reactive oxygen species, but not mitochondrial permeability transition in the apoptotic induction of human SK-Hep-1 hepatoma cells by shikonin. *Planta Med*, **69**, 1119-1124.

313. Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C. and Thomas, M. (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol*, **130**, 1910-1917.

314. Yang, G. Y., Liao, J., Kim, K., Yurkow, E. J. and Yang, C. S. (1998) Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis*, **19**, 611-616.

315. Wang, H. and Joseph, J. A. (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med*, **27**, 612-616.

316. Ormerod, M.G. (1999) *Flow Cytometry*. Springer-Verlag, Surrey, UK.

317. Waladkhani, AR and Clemens, MR (2003) Antioxidants as cancer therapies. In Watson, R. (ed.), *Functional foods and nutraceuticals in cancer prevention*. Iowa state press, Ames, Iowa, pp. 179-198.

318. Langner, E., Greifenberg, S. and Gruenwald, O. (1998) Ginger: History and use. *Advances in Therapy*, **15**, 25-44.

319. Kim, H. W., Murakami, A., Abe, M., Ozawa, Y., Morimitsu, Y., Williams, M. V. and Ohigashi, H. (2005) Suppressive effects of mioga ginger and ginger constituents on reactive oxygen and nitrogen species generation, and the expression of inducible pro-inflammatory genes in macrophages. *Antioxid Redox Signal*, **7**, 1621-1629.

320. Imanishi, N., Andoh, T., Mantani, N., Sakai, S., Terasawa, K., Shimada, Y., Sato, M., Katada, Y., Ueda, K. and Ochiai, H. (2006) Macrophage-mediated inhibitory effect of Zingiber officinale Rosc, a traditional oriental herbal medicine, on the growth of influenza A/Aichi/2/68 virus. *Am J Chin Med*, **34**, 157-169.

321. Imanishi, N., Mantani, N., Sakai, S., Sato, M., Katada, Y., Ueda, K., Terasawa, K. and Ochiai, H. (2004) Inducible activity of ginger rhizome (Zingiber offifinale Rosc.) on the mRNA expression of macrophage-inducible nitric oxide (NO) synthase and NO production in a macrophage cell line, RAW264.7 cells. *Am J Chin Med*, **32**, 727-735.

322. Aktan, F., Henness, S., Tran, V. H., Duke, C. C., Roufogalis, B. D. and Ammit, A. J. (2006) Gingerol metabolite and a synthetic analogue Capsarol inhibit macrophage NF-kappaB-mediated iNOS gene expression and enzyme activity. *Planta Med*, **72**, 727-734.

323. Katiyar, SK, Agarwal, R and Mukhtar, H (1996) Inhibition of tumor promotion in SENCAR mouse skin by ethanol extract of *Zingiber officinale* rhizome. *Cancer Research*, **56**, 1023-1030.

324. Nagasawa, H, Watanabe, K and Inatomi, H (2002) Effects of bitter melon (*Mamordica charantia* L.) or ginger rhizome (*Zingiber officinale Rosc*) on spontaneous mammary tumorigenesis in SHN mice. *The american journal of chinese medicine*, **30**, 195-205.

325. Watanabe, S, Kitade, Y, Masaki, T, Nishioka, M, Satoh, K and Nishino, H (2001) Effects of lycopene and sho-saiko-to on hepatocarcinogenesis in a rat model of spontaneous liver cancer. *Nutrition and cancer*, **39**, 96-101.

326. Rababah, TM, Hettiarachchy, NS and Horax, R (2004) Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *Journal of Agricultural and Food Chemistry*, **52**, 5183-5186.

327. Keum, YS, Kim, J, Lee, KH, Park, KK, Surh, YJ, Lee, JM, Lee, SS, Yoon, JH, Joo, SY, Cha, IH and Yook, JI (2002) Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. *Cancer Letters*, **177**, 41-47.

328. Kim, HW, Murakami, A, Nakamura, Y and Ohigashi, H (2002) Screening of edible japanese plants for suppressive effects on phorbol ester induced superoxide generation in differentiated HL-60 cells and AS52 cells. *Cancer Letters*, **176**, 7-16.

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PUBLICATIONS

1. Sun Z, Sood S, Li N, **Ramji D**, Yang P, Newman RA, Yang CS and Chen X (2006). Involvement of the 5-lipoxygenase/leukotriene A_4 hydrolase pathway in 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch, and inhibition of carcinogenesis by its inhibitors. *Carcinogenesis*, **27**, 1902-08

2. Huang MT, Liu Y, **Ramji D**, Sang S, Rosen RT, Ghai G, Yang CS and Ho, CT (2006). Effect of black tea theaflavins on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation: Expression of pro-inflammatory cytokines and arachidonic acid metabolism in mouse ear and colon carcinogenesis in Min (Apc+/-) mice. In Wang M, Sang S and Ho CT (eds.), *Herbs: Challenges in Chemistry and Biology of Herbs*. American Chemical Society, Washington, DC, pp. 314-25

3. Huang MT, Liu Y, **Ramji D**, Lo CY, Ghai G, Dushenkov S and Ho CT (2006). Inhibitory effects of black tea theaflavin derivatives on 12-*O*-tetradecanoyl phorbol-13-acetate-induced inflammation and arachidonic acid metabolism in mouse ears. *Molecular Nutrition in Food Research*, **50**, 115-122.

4. **Ramji D**, Sang S, Liu Y, Rosen RT, Ghai G, Ho CT, Yang CS and Huang, MT (2005). Effect of black tea theaflavins and related benzotropolone derivatives on 12-*O*-tetradecanoylphorbol-13-acetate-induced mouse ear inflammation and inflammatory mediators. In Shahidi, F. and Ho, C. (eds.), *Phenolic Compounds in Foods and Natural Health Products*. American Chemical Society, Washington, DC, pp. 242-253.

5. **Ramji, D**. (2004). Effect of black tea theaflavin derivatives on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation in mice. *Masters Thesis*, Rutgers University, NJ. pp 142.