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Regulation of Apoptosis, Invasion and Angiogenesis of Tumor Cells by Caffeic Acid Phenethyl Ester

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1. Introduction

Cancer is a multistage disease involving a series of events and generally occurs over an extended period. During this period, accumulation of genetic and epigenetic alterations leads to the progressive transformation of a normal cell into a malignant cell. Cancer cells acquire several abilities that most healthy cells do not possess: they become resistant to growth inhibition, proliferate without dependence on growth factors, replicate without limit, evade apoptosis, and invade, metastasize and support angiogenesis [1]. Unlike heart disease, death rates for cancer remained approximately the same in the United States from 1975 through 2002. Indeed, it is predicted that by 2020 approximately 15 million new cancer cases will be diagnosed worldwide and 12 million cancer patients will die [2].

Cancer is a disease characterized by uncontrolled growth and division of genetically altered cells and its emergence requires several elements, including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis [1, 3]. Cancer is thought to evolve along a multi-step process. Cancer cells are the descendants of a normal cell in which some kind of internal or external stress causes a change in its genetic code. This event is said to initiate the cell to a precancerous state. In a second stage, this precancerous cell divides in response to a promoting agent to produce daughter cells, and these daughter cells divide to produce more daughter cells, and so on. The genetic instabilities passed down through the generations finally result in one cell that no longer requires the promoting agent to stimulate its proliferation. A cancer cell is thus born with the ability to make proteins such as growth factors that stimulate proliferation. Finally in the third stage of carcinogenesis, progression, this cancer cell divides to produce daughter cells, which also divide, and soon there is a population of cancer cells with the ability to invade and metastasize [4].

It is now clear that cancer phenotypes result from the dysregulation of more than 500 genes at multiple steps in cell signaling pathways. This indicates that inhibition of a single gene product or cell signaling pathway is unlikely to prevent or treat cancer. However, most current anticancer therapies are based on the modulation of a single target [5, 6].

One of the most important findings to have emerged during the past three decades is that cancer is a largely preventable disease. Thus, people need to be educated about the risk factors for cancer and those that prevent the disease. As many as 90% of all cancers have been shown to be due to environmental/acquired factors such as tobacco, diet, radiation and infectious organisms, etc., and only the remaining 5–10% of cases are caused by internal factors such as inherited mutations, hormones, and immune conditions [7].

The ineffective, unsafe, and expensive monotargeted therapies have led to a lack of faith in these approaches. Therefore, the current paradigm for cancer treatment is either to combine several monotargeted drugs or to design drugs that modulate multiple targets. As a result, pharmaceutical companies have been increasingly interested in developing multitargeted therapies. Many plant-derived dietary agents, called nutraceuticals, have multitargeting properties. In addition, these products are less expensive, safer, and more readily available than are synthetic agents [5].

Prevention is better than cure and this is very true in case of cancer. Chemoprevention was defined as the administration of agents to prevent induction, to inhibit or to delay the progression of cancer [8], or as the inhibition or reversal of carcinogenesis at a premalignant stage [9]. Chemoprevention involves the use of synthetic or natural compounds to inhibit, slow, or reverse carcinogenesis. It is based on the hypothesis that the disruption of biological events involved in carcinogenesis will inhibit this process and can be applied to any stage of carcinogenesis. Chemoprevention utilizes appropriate pharmacological agents [10,11] or dietary agents, consumed in diverse forms like macronutrients, micronutrients, or nonnutritive phytochemicals [12–14]. It is estimated that from 10 to 80 percent of cancer patients use some form of natural compounds as a part of complementary medicine as part of their overall therapy without any real guidance. This explains the growing interest in using the natural compounds properly in the treatment of cancer.

Phytochemicals are one wide class of nutraceuticals found in plants, which are extensively researched by scientists for their health-promoting potential. Honey has a wide range of phytochemicals including polyphenols which act as antioxidants. Polyphenols and phenolic acids found in the honey vary according to the geographical and climatic conditions. Some of them were reported as a specific marker for the botanical origin of the honey. Considerable differences in both composition and content of phenolic compounds have been found in different unifloral honeys [15]. Terpenes, benzyl alcohol, 3, 5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl 3, 5-dimethoxy-4-hydroxybenzoate (methyl syringate), 3, 4, 5-trimethoxybenzoic acid, 2-hydroxy-3-phenylpropionic acid, 2-hydroxybenzoic acid and 1, 4-dihydroxybenzene are some of the phytochemicals ascribed for the antimicrobial activity of honey [16]. Among these phytochemicals, polyphenols were reported to have antiproliferative potential.

2. Active compounds in propolis

Polyphenolic compounds are widely distributed in the plant kingdom and display a variety of biological activities, including chemoprevention and tumor growth inhibition. Propolis and honey have been known to mankind from the remotest of ancient times and have been widely used by many cultures for different purposes. Propolis is a complex resinous mixture gathered from plants and used by honeybees in their hives as a general-purpose sealer and antibiotic. It is made up of a variety of polyphenolic compounds. Some of the isolated compounds have shown anti-inflammatory activity, carcinostatic, anti-carcinogenic activity and induction of apoptosis. Caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) are members of the polyphenolic compounds and present in high concentrations in medicinal plants and propolis. CAPE showed a wide variety of biological activities at non-toxic concentrations. It has shown antibacterial, anti-inflammatory, antioxidant, antitumor and anti-proliferative activities [17].

CAPE [2-propenoic acid, 3-(3,4-dihydroxy phenyl)-,2-phenethyl ester] (Fig.1) is an active component of propolis with a variety of biological activities. CAPE has been used in folk medicine as a potent antibacterial, anti-inflammatory, antioxidant, antitumor and antiproliferative with a wide variety of biological and pharmacological activities at non-toxic concentrations in a mammal's organs [18].

Figure 1. Structure of caffeic acid phenethyl ester (CAPE)

CAPE is chemopreventive against intestinal, colon and skin cancer, and also has been shown to decrease the formation of preneoplastic hepatic lesions when administered in a rat model of liver carcinogenesis [19-21], but the mechanism of these properties is not completely understood. Recently, CAPE, in a concentration dependent fashion, was shown to inhibit MCF-7 (hormone receptor positive, HR+) and MDA-MB-231 (a model of triple negative BC (TNBC)) tumor growth, either *in vitro* or *in vivo* without much effect on normal mammary cells [22]. At the same time, CAPE was found to cause pronounced changes in bCSC characteristics manifested by inhibition of self renewal, progenitor formation, clonal growth in soft agar, and concurrent significant decrease in CD44 content, all signs of decreased malignancy potential [23]. Besides CAPE, other caffeic acid esters in propolis may have biological effects.

Here we will focus on CAPE and its biological effects against cancer *in vitro* and *in vivo*. Specifically, we will discuss how CAPE can modulate inflammatory pathways and thus affect the survival, proliferation, invasion, angiogenesis, and metastasis of the tumor.

2.1. Regulation of inflammatory pathways by CAPE

Inflammation is a localized reaction of tissue to infection, irritation, or other injury. Inflammation is a necessary response to clear bacterial and viral infections, repair tissue insults, and suppress tumor initiation/progression. However, when inflammation persists or control mechanisms are dysregulated, diseases such as cancer can develop. Interestingly, inflammation functions at all stages of tumor development: initiation, promotion and progression including metastasis. During the initiation phase, inflammation induces the release of a variety of cytokines and chemokines that promote the activation of inflammatory cells and associated factors. This causes further oxidative damage, DNA mutations, and other changes in the tissue microenvironment, making it more conducive to cell transformation, increased survival, and proliferation [24]. At the molecular level, inflammation, transformation, survival and proliferation are regulated by the proinflammatory transcription factor Nuclear Factor- κ B (NF- κ B), a family of ubiquitously expressed transcription factors. NF- κ B regulates the expression of genes involved in the transformation, survival, proliferation, invasion, angiogenesis and metastasis of tumor cells [25].

TNF- α is also one of the prime signals that induces apoptosis in many different types of cells. Whereas acute activation of NF- κ B may be therapeutic, chronic activation may lead to the development of chronic inflammation, cancer and other chronic diseases. There is a strong association between chronic inflammatory conditions and cancer specific to the organ. Epidemiological evidence points to a connection between inflammation and a predisposition for the development of cancer, i.e., long-term inflammation leads to the development of dysplasia. Various factors are known to induce chronic inflammatory responses that further cause cancer. These include bacterial, viral, and parasitic infections (e.g., *Helicobacter pylori*, Epstein-Barr virus, human immunodeficiency virus, flukes, schistosomes) and chemical irritants (i.e., tumor promoters). Active NF- κ B has now been identified in tissues of most cancer patients, including those with leukemia and lymphoma and cancers of the prostate, breast, oral cavity, liver, pancreas, colon and ovary [26].

In the resting stage, NF- κ B resides in the cytoplasm as a heterotrimer consisting of p50, p65, and the inhibitory subunit I κ B α . On activation, the I κ B α protein undergoes phosphorylation, ubiquitination, and degradation. p50 and p65 are then released, are translocated to the nucleus, bind specific DNA sequences present in the promoters of various genes, and initiate their transcription. A number of proteins are involved in the NF- κ B signaling pathway. Because of the relevance of the NF- κ B signaling pathway in cancer, this pathway has been proven to be an attractive target for therapeutic development. Active NF- κ B complexes can contribute to tumorigenesis by regulating genes that promote the growth and survival of cancer cells, during the cell cycle. NF- κ B has ability to regulate

the G1-phase expression of key proto-oncogenes is subject to regulation by the integrated activity of IkappaB kinase (IKK)alpha, IKKbeta, Akt and Chk1. The coordinated binding of NF-κB subunits to the Cyclin D1, c-Myc and Skp2 promoters is dynamic with distinct changes in promoter occupancy and RelA(p65) phosphorylation occurring through G1, S and G2 phases, concomitant with a switch from coactivator to corepressor recruitment. Akt activity is required for IKK-dependent phosphorylation of NF-κB subunits in G1 and G2 phases, where Chk1 is inactive. However, in S-phase, Akt is inactivated, while Chk1 phosphorylates RelA and associates with IKKalpha, inhibiting the processing of the p100 (NF-κB2) subunit, which also plays a critical role in the regulation of these genes. This reveals a complex regulatory network integrating NF-κB with the DNA-replication checkpoint and the expression of critical regulators of cell proliferation [27]. Thus, its inhibition could be a novel approach to breaking the vicious cycle of tumor cell proliferation [28-30]. More than 700 inhibitors of the NF-κB activation pathway have been reported, including antioxidants, peptides, small RNA/DNA, microbial and viral proteins, small molecules, and engineered dominant-negative or constitutively active polypeptides [31,32].

Caffeic acid phenethyl ester has been shown to suppress NF-κB activation by suppressing the binding of the p50-p65 complex directly to DNA [33-35]. The molecular basis of CAPE action was elucidated by Natarajan et al. [36]. Since NF-κB has a role in these activities, they examined the effect of CAPE on this transcription factor in an exhaustive manner. They preincubated the U-937 cells with CAPE at various concentrations for 2 hours before treating with TNF (0.1 nM) for 15 minutes. CAPE inhibited the TNF-dependent activation of NF-κB in a dose-dependent manner with maximum effect occurring at 25 μg/mL. NF-κB activation induced by the phorbol ester, phorbol-12-myristate 13-acetate (PMA), ceramide, okadaic acid and hydrogen peroxide was also inhibited by CAPE. It prevented the translocation of the p65 subunit of NF-κB to the nucleus without affecting the TNF-induced IκBα degradation. It did not show any inhibitory effects on the other transcription factors like AP-1, TFIID and oct-1. With these findings they concluded that CAPE is a potent and a specific inhibitor of NF-κB activation and this may provide the molecular basis for its multiple immunomodulatory and antiinflammatory activities of CAPE.

Abdel-Latif et al.[37] have demonstrated for the first time that CAPE is a major component of propolis, modulates *H. pylori*-induced NF-κB, AP-1 DNA binding activity and COX-2 expression in gastric epithelial cells. In addition, CAPE reduced TNF-α and IL-8 levels and suppressed the proliferative response of AGS cells to *H. pylori*. They found also that pretreatment of gastric epithelial cells with CAPE upregulated IκB-α levels and prevented nuclear translocation of NF-κB/ p65 in *H. pylori*-treated AGS cells. NF-κB is present in the cytosol in an inactive state bound to the inhibitory IκB protein. *H. pylori* infection of gastric epithelial cells results in phosphorylation and degradation of the IκB, thus allowing nuclear translocation of NF-κB.

It is mechanistically proven that inflammation produces reactive oxygen species (ROS) and reactive nitrogen species (RNS). In particular, ROS and RNS lead to oxidative damage and nitration of DNA bases, which increases the risk of DNA mutations and further leads to cancer. Nitric oxide (NO) is associated with inflammatory reaction and is produced by

inducible nitric oxide synthase (iNOS) in certain cells activated by various proinflammatory agents. NO acts as a host defense by damaging membranes of pathogenic bacteria and as a regulatory molecule with homeostatic activities. However, excessive production of NO is pathogenic for host tissue itself because NO, as a reactive radical directly damages functions of normal tissue. Thus, effective inhibition of NO accumulation by inflammatory stimuli represents a beneficial therapeutic strategy [38,39].

Nagaoka et al. [40] reported that CAPE possesses potent NO inhibitory activities and suggested that the NO inhibitory effect can directly correlate with anti-inflammatory properties of the Netherlands propolis. They suggested that the active principles of the Netherlands propolis, i.e., CAPE and its analogues, should block the activation of iNOS through the suppression of NF- κ B activation and resulted in potent NO inhibition.

We investigated the anti-inflammatory and antioxidant potential of CAPE on a tumor cell line (ZR-75-1). We found that CAPE at the concentration of 15 μ M inhibited NO production by (> 47%) compared to NO level of untreated tumor cells ($P < 0.05$). In addition, superoxide dismutase (SOD) was at the highest level in the maintained basal tumor culture cell supernatant (231.9 $\pm$ 4.2 μ U/L). This level reduced intensively to (169.3 $\pm$ 3.7 μ U/L), in the CAPE-treated culture cells, which was significantly less than the level in untreated cells ($P < 0.001$). On the other hand, malonaldehyde (MDA) level, which is considered to be an important parameter for the oxidative damage determination, was inhibited to (17.3 $\pm$ 2.3 μ mol/L) in the CAPE-treated cells when compared with untreated tumor cells (23.8 $\pm$ 2.5 μ mol/L), but was not statistically significant (Table 1).

Group Parameters	NO $\cdot$ (μ mol/L)	SOD (μ U/ L)	MDA (μ mol/L)
I	19.6 $\pm$ 1.9	231.9 $\pm$ 4.2	23.8 $\pm$ 2.5
II	10.2 $\pm$ 1.3	169.3 $\pm$ 3.7	17.3 $\pm$ 2.3
P	< 0.05	< 0.001	N.S

(I). Untreated tumor cells , (II). Treated tumor cells at 15 μ M CAPE.

Table 1. Nitric oxide (NO $\cdot$), Superoxide dismutase (SOD) and Malondialdehyde (MDA) values in culture supernatant.

2.2. Regulation of tumor cell development by CAPE

Under normal physiological conditions, the human body maintains homeostasis by eliminating unwanted, damaged, aged, and misplaced cells. Homeostasis is carried out in a genetically programmed manner by a process referred to as apoptosis (programmed cell death). Cancer cells are able to evade apoptosis and grow in a rapid and uncontrolled manner. One of the most important ways by which cancer cells have gained this ability is through mutation in the p53 tumor suppressor gene. Without a functional p53 gene, cells lack the DNA-damage-sensing capability that would normally induce the apoptotic cascade [41-43].

A complex set of proteins, including caspases, proapoptotic and antiapoptotic B cell lymphoma (Bcl)-2 family proteins, cytochrome c, and apoptotic protease activating factor (Apaf)-1, execute apoptosis either by an intrinsic or extrinsic pathway. The intrinsic pathway is mitochondria dependent, whereas the extrinsic pathway is triggered by death receptors (DRs). Some antiapoptotic proteins such as Bcl-2 and B cell lymphoma extra large (Bcl-xL) and survivin are overexpressed in a wide variety of cancers. Therefore, selective downregulation of antiapoptotic proteins and upregulation of proapoptotic proteins and p53 in cancer cells offer promising therapeutic interventions for cancer treatment [44,45].

We tested the effect of CAPE on the viability of human breast cancer ZR-75-1 cells derived from a malignant ascitic effusion in a 63 year-old, white female with infiltrating ductal carcinoma [46]. CAPE induced a significant inhibitory effect on the growth and viability of tumor cells in vitro. We observed that this inhibitory action was highly dosage and time-dependent. The maximum inhibitory action was obtained at 15 μ M (Figure 2A) on culture media.

Figure 2. CAPE induced ZR-75-1 cell death (A); cell viability was observed in tumor cells treated with different CAPE concentrations (5, 10, 15 and 20 μ M), while (B) cells showed maximum alteration of viability at 48 hrs. of incubation, indicating that CAPE induced cell death in a dose and time dependent manner. The results shown in the histogram were the mean $\pm$ S.D. Assay was performed in 12 – well plate (2×10^6) cells/well, using trypan blue dye for viability detection.

On the other hand, after 48 hours of administration, the percentage of cell death increased significantly to 49.6 ± 6.9 (Figure 1B). No further changes were observed after 72 hours of treatment. To investigate the induced effect of apoptosis on cell viability, we analyzed the DNA fragmentation using DAPI. The nuclear structure exhibited condensation and fragmentation of some nuclei that were caused by 15 μ M CAPE at 48 hours. Apoptotic cells count significantly increased by 27% as compared to control tumor cells ($P < 0.01$) (Figure 3B) [47].

Additionally, the microscopic examination did not reveal any signs of morphological changes after 12 hours of administration. However, a scattered retraction of the monolayer, vacuoles, and the granulation of the cytoplasm were observed after 24 hours. The

alterations were further aggravated after 48 hours. The cells were rounded up. Later, they became phase-dense and formed floating aggregates, which gradually increased in size and most of the cells were detached from the flasks. The cell membranes burst, which was followed by a gradual decrease of the cell count. These changes were not reversible. Transferring the cells into a fresh medium did not alter their state (Figure 4) [47].

Figure 3. Effect of CAPE on expression of apoptotic cells. (A) ZR-75-1 cells without treatment. (B) Cells were treated with 15 μ M CAPE for the indicated time of 48 hrs. Condensation and apoptotic bodies were examined by immunofluorescence microscopy. Magnification, X20.

Figure 4. CAPE induced cell detaching (A) ZR-75-1 cells kept without treatment during experiments. (B) 15 μ M CAPE treatment after 48 hrs. Morphology changes in cells were examined by phase-contrast microscopy. Magnification, X40.

2.3. Tumor growth inhibition *in vivo* by CAPE

The effects of CAPE on the survival of mice bearing tumor are shown in figure 5. The median survival time for the untreated group of mice was 21 days. On the other hand, the group of mice bearing tumor and treated with 10mg/kg S.C/ every 5 days had a median survival time of 29 days. Two mice were completely cured. The median survival time of the group treated with 15mg/kg S.C/ every 5 days was found to be 43 days and 3 mice were completely cured. However, those mice treated with 5mg /kg S.C/ every 5 days did not show any remarkable changes in their survival percentage. It has also been observed that giving treatment more than once a week caused sores and increased irritability in the mice, vehicle (1:1/DMSO: NaCl).

We studied the effect of CAPE (15mg/kg S.C/ every 5 days) on the growth of transplanted Ehrlich carcinoma into Swiss mice. The solid tumor volume showed a reduced rate in CAPE-treated mice and appreciably smaller volume (1.9 ± 0.46) mm³, with respect to the untreated group (3.7 ± 0.82) mm³. This value was significant ($P < 0.01$). The difference was observed from the beginning of tumor measurement i.e. since the 6th day after tumor implantation to the host, and was maintained until the end of observation (Figure 6). This finding is one of the characteristic effects of anti-tumor drugs. It is also in accordance with

the other findings, which suggest that subcutaneous administration of an aqueous crude water-soluble propolis (CWSP) resulted in marked regression of transplanted tumors [48].

Figure 5. Effect of CAPE treatment on the time of survival for mice bearing solid tumor at different doses compared with untreated group.

Figure 6. Effect of CAPE (15mg/kg S.C), once/week and twice/week treatment on tumor volume against untreated group of mice. Data represented as mean $\pm$ S.D.

The percentage of apoptotic cells with hypodiploid DNA content was determined from DNA histograms. Untreated tumor-bearing mice showed a peak pattern which represented proliferative and high metastatic tumor activity (Figure 7A). However the mice which had their tumor treated at 15mg/kg S.C did not alter the relative size of the peak, but induced a significant parallel shift to less-intense fluorescence (D- area). This decrease in the intensity and shift may be termed as nuclear apoptosis and fragmentation (Figure 7B).

Figure 7. The value in the tumor apoptosis histogram was higher in mice-bearing tumors, which were treated with CAPE (15mg/kg/S.C). (B) as compared to mice-bearing tumors untreated with CAPE (A). The apoptosis was identified by PI staining (D-area) with increased DNA fragmentation [47].

Chung et al., [49] showed that both CA and CAPE selectively inhibit matrix metalloproteinases (MMP) 2 and 9. CAPE inhibited strongly MMPs 2 and 9 with IC_{50} of 2–5 μ M, while CA required 10–20 μ M for such inhibition. In contrast, MMPs 1, 3, 7 and Cathepsin-K were not completely inhibited by either of them. CA and CAPE had a dose-dependent inhibitory effect on the proliferation of HEPG2 cells. In HepG2 cells, CA at the concentration of 200 μ g/mL reduced the viability to 61%, while CAPE, at 10 times lower concentration, inhibited the viability to 72% as compared to the respective controls. CAPE and CA suppressed the MMP 9 expression in HepG cells exposed to phorbol 12-myristate 13-acetate (PMA). They also confirmed that CA (20 mg/kg) and CAPE (5mg/kg) repressed the growth of HepG2 tumor xenografts in nude mice as well as liver metastasis when administered subcutaneous or orally. Finally they concluded their observation that CA and its derivative CAPE: (1) inhibited the enzymatic activity of MMP-9 that plays an important role in cancer invasion and metastasis, (2) blocked the invasive potential through the suppression of MMP-9 gene transcription by inhibiting NF- κ B function in PMA-stimulated HepG2 cells and (3) suppressed the growth of HepG2 cell xenografts in nude mice. Therefore, these two drugs were reported as strong candidates for treatment of cancer and metastasis via dual mechanisms (dual inhibition of metastasis-specific enzyme activity and gene transcription) [50].

2.4. Regulation of tumor cell proliferation by CAPE

Dysregulated proliferation is one of the major characteristics of tumorigenesis. In normal cells, proliferation is regulated by a delicate balance between growth signals and antigrowth signals. Cancer cells, however, acquire the ability to generate their own growth signals and become insensitive to antigrowth signals [51]. Their growth is controlled by cell cycle regulators at the G1/S-phase boundary, in the S phase, and during the G2/M phases of the cell cycle. A precise set of proteins called cyclins and cyclin-dependent kinases (CDKs) control the progression of cell cycle events. Whereas cyclin binding is required for CDK activity, CDK inhibitors (CKIs) such as p21 and p27 prevent CDK activity and prevent cell cycle progression. The G1-to-S-phase transition also requires cellular v-myc myelocytomatosis viral oncogene homolog (c-Myc), and inhibition of c-Myc expression leads to growth arrest [52,53].

The expression of c-Myc in turn is regulated by cdc25, a phosphatase that activates CDKs. The well-characterized tumor suppressor p53 has been implicated in controlling the G1-to-S-phase transition and in blocking cell cycle progression at the G1 phase in response to DNA damage [54]. A number of genes controlling cell cycle progression, including the CKI p21, are transcribed in a p53-dependent manner [55,56].

Rb is a tumor suppressor retinoblastoma protein that, like p53, functions as a negative regulator of cell growth [57]. Rb inactivation or deletion has been found in many cancers, including retinoblastomas and carcinomas of the lung, breast, bladder, and prostate. By binding to and inhibiting transcription factors such as elongation 2 factor (E2F), which are necessary for S-phase entry, Rb is believed to inhibit cell cycle progression [58]. On the other hand, phosphorylation of Rb (pRb) by CDK/cyclin complexes results in the release of active E2F species to stimulate the transcription of genes involved in DNA synthesis and S-phase progression [59,60].

Currently, a number of inhibitors based on cell cycle regulators, including nutraceuticals, are being developed as therapeutic intervention for cancer prevention. Nutraceuticals have been shown to have potential in cancer prevention for halting cell cycle progression by targeting one or more steps in the cell cycle. Most nutraceuticals prevent the transition of cancer cells from the G1 to S phase. Some of these nutraceuticals act through p53 and some through Rb. Acetyl-keto-beta-boswellic acid was shown to arrest colon cancer cells at the G1 phase, which was associated with decreases in cyclin-D1, cyclin-E, CDK-2, CDK-4, and pRb and an increase in p21[61].

In Ehrlich ascites tumor cells, acetoxychavicol acetate was shown to stimulate the accumulation of tumor cells in the G1 phase of the cell cycle, which was accompanied by a decrease in pRb and an increase in Rb [62]. β -Escin, a triterpene saponin, induced cell cycle arrest at the G1/S phase by inducing p21 and reducing pRb in a p53-independent manner in HT-29 human colon cancer cells [63]. In gastric cancer cells, curcumin was shown to suppress the transition of cells from the G1 to S phase, which was accompanied by a decrease in cyclin-D1 and p21-activated kinase 1 activity [64].

The therapeutic goal of cancer treatment is to induce apoptotic death of cancer cells rather than necrosis due to the deleterious consequences of the latter, which include leakage of lysosomal enzymes to the extracellular media and spawning a substantial inflammatory reaction. Several investigators have demonstrated that CAPE has an anti-proliferative effect and an apoptosis inducing effect against various tumor cell lines. Cavaliere et al., showed that CAPE treatment increased the percentage of cells in G0/G1 and decreased the percentage of cells in S and G2/M phase in addition to its ability to inhibit DNA, RNA, and protein synthesis, thus delaying cell cycle progression to G2/M phase. CAPE also caused high levels of apoptotic cell death of 77.1%, with no signs of significant necrosis in PL104 cells [65]. Also, Wang et al., demonstrated that CAPE treatment was associated with a strong inhibition of proliferation in a dose- and time-dependent manner, along with induction of G0/G1 arrest and apoptosis in HCT116 cells [66].

2.5. Regulation of tumor cell invasion by CAPE

Tumor cell invasion and metastasis are interrelated processes involving cell growth, cell adhesion, cell migration, and proteolytic degradation of tissue barriers such as the extracellular matrix and basement membrane. Several proteolytic enzymes, including MMPs (chiefly MMP-2 and MMP-9) [67,68] and intercellular adhesion molecule (ICAM; chiefly ICAM-1), participate in the degradation of these barriers [69,70]. A number of studies in lung, colon, breast, and pancreatic carcinomas have demonstrated overexpression of MMPs in malignant tissues compared with adjacent normal tissues [71-78]. Apart from MMPs, cysteine proteases [79] and serine proteases [80] such as urokinase-type plasminogen activator (u-PA) have also been involved in the invasion and metastasis of cancer cells. Since both u-PA and u-PA receptor (u-PAR) contain binding sites for NF- κ B and activator protein (AP)-1 in their promoter regions [81-83], inhibition of these transcription factors will eventually result in the inhibition of u-PA–u-PAR complex and subsequent suppression of invasive behavior.

Furthermore, Hwang et al. [84] investigated the effect of CAPE on tumor invasion and metastasis in HT 1080 fibrosarcoma cells by determining the regulation of matrix metalloproteinases (MMPs). HT 1080 cells were treated with increasing concentration of CAPE and the m-RNA transcripts of MMP-2 and MMP-9 were analyzed using semi-quantitative RT-PCR. Both MMP-2 and 9 proteins levels were significantly suppressed in a dose dependent manner. Gelatin zymography also indicated constitutively expressed MMP-2 and 9 proteins in HT 1080 cells, which gradually reduced after treating with CAPE. To further corroborate the down regulation of MMP-2, activation studies of pro-MMP2 were performed using organomercuric compound, 4-aminophenylmercuric acetate (APMA), and the result indicated the down regulation of MMP-2 by CAPE. It has been shown that mRNA levels of tissue inhibitor of matrix metalloproteinases (TIMPs) and membrane type-matrix metalloproteinases (MT-1 MMPs) were also reduced significantly. CAPE also inhibited the cell invasion, cell migration and colony formation of tumor cells. Thus CAPE acts as a vital antimetastatic agent, by inhibiting the metastatic and invasive potential of malignant cells.

2.6. Regulation of tumor cell angiogenesis by CAPE

Angiogenesis, the process during which new blood vessels are formed from preexisting ones, can be classified as either physiological or pathological. Physiological angiogenesis provides a driving force for organ development in ontogeny, is necessary for ovulation, and is a prerequisite for wound healing; pathological angiogenesis occurs during tumor growth at primary and metastatic sites [85]. The angiogenic cascade during tumor development consists of the release of angiogenic factors, binding of angiogenic factors to receptors on endothelial cells (ECs), EC activation, degradation of the basement membrane by proteases, and migration and proliferation of ECs. Adhesion molecules then help to pull the sprouting blood vessels forward, and ECs are finally organized into a network of new blood vessels [86].

The signaling pathway governing tumor angiogenesis is exceedingly complex, involving various angiogenic mediators. The major signaling mediators include VEGF, platelet-derived growth factor, fibroblast growth factors (FGFs), epidermal growth factor, ephrins, angiopoietins, endothelins, integrins, cadherins, and notch [87].

Group Parameters	Normal Group n = 10	Tumor Group n = 10	Treated Group n = 10	P ₁	P ₂
MMP-9 (ng/ml)	130.9	181.9	142.1	P< 0.001	P< 0.01
Median Range	79.6 – 166.4	88.2 – 216.1	84.5 – 196.3		
Endostatin (ng/ml)	2.2	1.4	1.9	P = 0.01	P< 0.01
Median Range	1.1 – 6.3	1.0 – 5.4	1.3 – 9.3		

P1 Tumor group Vs Normal group

P2 Treated group Vs Tumor group

Table 2. Levels of MMP-9 and Endostatin serum in normal mice group, tumor bearing group and treated tumor group.

We found that the untreated mice bearing Ehrlich tumor elicited a highly significant increase of serum MMP-9 level (181.9 ng/ml), which was reduced (142.1 ng/ml) in mice treated with CAPE at a dose of 15mg/kg ($P < 0.01$) close to the normal mice serum level. However, in the untreated mice bearing tumor serum endostatin (sE) was significantly lowered (1.4 ng/ml) compared with the normal mice. In CAPE-treated mice serum endostatin level was significantly higher (1.9 ng/ml) than the serum level in the untreated group (Table 2)[47]. On the other hand, there was a negative correlation between (sMMP-9 and sE) and the total white blood cells (WBCs), hemoglobin (HB) and the platelet count of mice (Table 3)[47]. Based on these findings, we concluded that; the endogenous inhibitor of the angiogenic serum (endostatin) has been shown to be overexpressed, significantly higher in treated mice compared to untreated mice (1.9 ng/ml), and nearly to the value of normal serum mice (Table 2). These findings may help to utilize endostatin itself in the therapy. It also indicates that

CAPE has the potential of an anti-metastatic agent. It may mediate CAPE effects by inhibiting the cell proliferation. The findings of this study are in accordance with Schuch et al. [88] who, claimed that endostatin microbeads significantly inhibit the growth of subcutaneous choloromas in SCID mice as compared to control mice. On the other hand, MMP-9 and endostatin did not correlate with the white blood cells (WBCs), hemoglobin (HB) and platelet count (Table 3). It may be concluded that MMP-9 and endostatin are independent factors.

	HB	WBCs Count	Platelets Count
MMP-9	R= 0.11, >0.05	R= 0.19, >0.05	R= 0.24, >0.05
Endostatin	R= 0.09, >0.05	R= 0.1, >0.05	R= 0.04, >0.05

Table 3. Correlation between investigated angiogenic factors and, hemoglobin (HB), white blood cells count (WBCs) and platelets count in tumor treated mice.

3. Conclusion

These findings obtained suggest that CAPE is a potent agent, which has antioxidant properties. *In vitro* findings support that CAPE could be potentially useful in the control of tumor cell proliferation as well as, an apoptotic-inducing agent. Furthermore, CAPE exhibits anti-metastatic and anti-angiogenic properties. CAPE could be potentially useful in the control of tumor growth in experimental models. Its action is accompanied by the shifting and elevating of the angiostatic and inhibiting angiogenic factors. Finally, it has been demonstrated that CAPE has many biological and pharmacological properties with predictive future applications in human clinical trials.

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