

# Multiple Molecular Targets of *Antrodia camphorata*: A Suitable Candidate for Breast Cancer Chemoprevention

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## 1. Introduction

Data from cancer registries reported that cancer incidence is increasing every year and cancer claimed the second leading causes of death worldwide, surpassed only by cardiovascular disease. National Cancer Research Institute classified that the breast cancer ranks second as a cause of cancer death in women, followed by lung cancer. Globally, more than 1.1 million women are diagnosed with breast cancer every year at the same time nearly 410, 000 women are queued for die due to the breast cancer (Cancer factors and Figures, 2010, American Cancer Society). The incidence of breast cancer varies greatly around the world. Lowest breast cancer incidence was observed in less developed countries, whereas highest in the more developed countries. In United States alone, annually more than 240, 000 women are diagnosed breast cancer and nearly 180, 000 women are diagnosed with the most deadly invasive breast cancer. It is also notable that about 1 in 8 women in the United States (12%) will develop invasive breast cancer of her life time. In 2010, an estimated 207, 090 new cases of invasive breast cancer were expected to be diagnosed in women in the U.S., along with 54, 000 new cases of non-invasive (*in situ*) breast cancer. In addition, approximately 2000 men are expected to be diagnosed with invasive breast cancer in 2010. The survival rate for women diagnosed with localized breast cancer (cancer that has not spread to lymph nodes or other location in outside the breast) is 98%. If the invasive cancer that has spread to nearby or distant lymph nodes or organs, the five years survival is 84% or 23%, respectively. However, the surprising result is the five year relative survival for female breast cancer patients has improved from 63% in the early 1960's to 90% today.

Women is primary risk factor for developing breast cancer, because, women's naturally have more breast cells than men. The main reason for develop more breast cells in women due to the constant exposure of growth-promoting effects of the female hormones especially, estrogen and progesterone. Aside from being female, age is the most important risk factor for breast cancer. Potentially modifiable risk factors include weight gain after age 18. About 1 out of 8 invasive breast cancer are found in women younger than 45, while 2 out of 3 invasive breast cancer are common in women age 55 or older. Many studies have shown that being over weight adversely affects survival for postmenopausal women with breast

cancer risk and those women who are more physically active and less to die from the disease than women who are in active. The actual fact that only 20-30% of women are diagnosed with breast cancer has significant family history of breast cancer. However, a women's risk of breast cancer approximately doubles if she has a first-degree relative (mother, sister, and daughter) who has been diagnosed with breast cancer. Apart from the family history of breast cancer, personal breast cancer history also a major risk factor for further onset. For an example, A women with cancer in one breast has a 3 to 4-fold increased risk of developing a new cancer in the other breast or other part of the same breast. This is unlike from first breast cancer recurrence.

Mammography and ultrasonography are still the most effective for women with non-dense and dense breast tissues, respectively. Additionally, MRI, lymphatic mapping, the nipple-sparing mastectomy, partial breast irradiation, neoadjuvant systemic therapy, and adjuvant treatment are promising for subgroups of breast-cancer patients. Although, there few drugs are commercially available, the well known tamoxifen can be offered for endocrine-responsive disease, aromatase inhibitors are increasingly used. Assessment of potential molecular targets is now important in primary diagnosis. Tyrosine kinase inhibitors and other drugs with anti-angiogenesis and cancer cell metastasis inhibitors are currently undergoing preclinical investigations. Recent study show the experimental drug iniparib ultimately shrank tumors and increased the time they took to progress, in addition iniparib prolonging survival in women with what's known as triple-negative breast cancers. This type of breast cancer lacks receptors for estrogen and progesterone and doesn't have large quantities of HER-2/neu protein, which the most successful cancer therapies target. This means that may currently available drugs simply won't affect it. Therefore, new class of chemotherapeutic drug that can potentially inhibit the growth of estrogen-nonresponsive breast cancer are highly warranted.

The indigenous Taiwanese medicinal mushroom *A. camphorata* (*Syn, Antrodia cinnamomea; Taiwanofungus camphoratus*), locally known as "Niu Chang Chih" is a parasitic fungus grown in the inner cavity on the aromatic tree *Cinnamomum kaneirai* Hay (Lauraceae). This species is endemic to Taiwan and has been widely used as a Chinese folk medicine and functional food. In Taiwanese culture, it is believed that *Niu Chang Chih* is a valuable gift from the haven. Thereby, it claimed "National treasure of Taiwan". This species was first published by Zang & Su (1990). Dr. Su, a residential chemist, knew *Niu Cheng Chih*" very well from his chemical studies of various medicinal fungus (Wu et al., 1997) *A. camphorata* is starting to attract interest due to their abundant bioactive phytochemicals including, triterpenoids, flavonoids, polysaccharides, maleic/succinic acid, benzenoids and benzoquinone derivatives. The current scientific world's particular interest in *A. camphorata* and its curative properties originated from the realm of traditional practice. Till the dates there are more than three hundred scientific reports were published regarding the therapeutic potential of *A. camphorata* or it's derived pure compounds. Both the fruiting bodies and mycelium of *A. camphorata* has been shown to exhibit a wide range of health promoting benefits for the hepatic, neurological and cardiovascular systems. It also shown to inhibit variety of inflammation, viral infection, oxidative stress, atherosclerosis and the growth of a variety of cancer cells (Ao et al., 2009; Geethangili & Tzeng, 2009).

### 1.1 Clinical studies on *A. camphorata* as an adjuvant therapy for cancer

The anticancer potential of *A. camphorata* was recognized as early as 2002, when it was shown to inhibit proliferation and enhanced apoptosis in cultured human premyelocytic

leukemia HL-60 cells (Hseu et al., 2002; 2004). Thereafter extensive studies have verified the cancer-preventing or anti-cancer properties of *A. camphorata* and their derived pure compounds in various murine models of human cancer cell lines. Both the fruiting bodies and mycelium of *A. camphorata* have potent anti-proliferative activity against various cancers *in vitro* and *in vivo* (Table. 1). Its chemopreventive action against various cancer cells *via* modulating multiple signaling pathways at various cellular levels, the ultimate outcomes of which are apoptosis, cell cycle arrest, growth inhibition, anti-angiogenesis and inhibition of metastasis. Figure 1, illustrating the molecular targets modulated by *A. camphorata*.

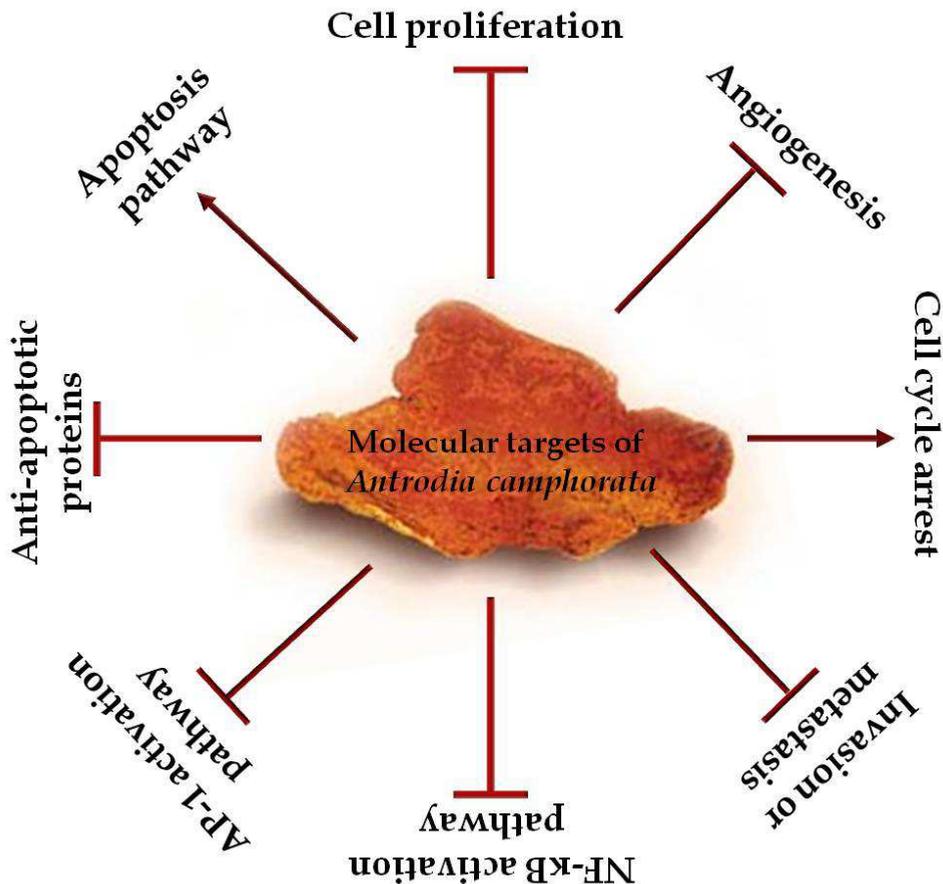


Fig. 1. Molecular targets of *A. camphorata* on various cancers

Agent	Mechanism	Target	Model	Cell lines or animal
<i>A. camphorata</i>	Cell cycle arrest	Cell cycle regulatory proteins	<i>In vitro</i>	PC3 & LNCap human prostate cancer cells
<i>A. camphorata</i>	Inhibits tumorigenesis	COX-2 and MDR-1 activity	<i>In vitro</i>	HepG2 human liver cancer cell
<i>A. camphorata</i>	Inhibits tumorigenesis	COX-2 and MDR-1 activity	<i>In vitro</i>	HepG2 human liver cancer cell
<i>A. camphorata</i>	Immunomodulation	HER-2/neu activity	<i>In vivo</i>	Inbreed female C3H/HeN mice
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	MDA-MB-231 breast cancer cell*
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	HepG2 human liver cancer cell
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	HL-60 human premyelocytic leukemia
<i>A. camphorata</i>	Cell cycle arrest	Cell cycle regulatory proteins	<i>In vitro</i>	T24 bladder cancer cell
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	MCF-7 human breast cancer cell*
<i>A. camphorata</i>	Inhibits proliferation	Cytokines activity	<i>In vitro</i>	HepG2, MCF & colon-205 cancer cells*
<i>A. camphorata</i>	Induce apoptosis	Ca <sup>2+</sup> activity	<i>In vitro</i>	PC3 human prostate cancer cell
<i>A. camphorata</i>	Induce apoptosis	Ca <sup>2+</sup> and MAPK activity	<i>In vitro</i>	OC2 human oral cancer cell
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	HepG2 and PLC/PRF/5 human cancer cells
<i>A. camphorata</i>	Induce apoptosis	Ca <sup>2+</sup> /calpain activity	<i>In vitro</i>	HepG2 human liver cancer cell
<i>A. camphorata</i>	Induce apoptosis	Caspase activity	<i>In vitro</i>	A549 & NSCLC human lung cancer cells
<i>A. camphorata</i>	Inhibits metastasis	KF-	<i>In vitro</i>	PLC/PRF/5 human liver cancer cell
<i>A. camphorata</i>	Induce senescence	Cell cycle regulatory proteins	<i>In vitro</i>	RT4, TSGH-8301 and T-4 carcinomas
<i>A. camphorata</i>	Inhibits metastasis	NF-	<i>In vitro</i>	MDA-MB-231 human breast cancer cell*
<i>A. camphorata</i>	Cell cycle arrest	Cell cycle regulatory proteins	<i>In vitro</i>	MDA-MB-231, athymic nude mice (BALB/c-nu)*
<i>A. camphorata</i>	Induce apoptosis	Fas activity	<i>In vitro</i>	HepG2 human liver cancer cell
Triterpenoids	Induce apoptosis	Caspase activity	<i>In vitro</i>	A549 & NSCLC human lung cancer cell
Polysaccharides	Inhibits angiogenesis	VEGF expression	<i>In vitro</i>	Bovine aortic endothelial cells
Polysaccharides	Inhibits angiogenesis	VEGF expression	<i>In vitro</i>	HL-60 & HUVECs
Polysaccharides	Inhibits tumorigenesis	Cytokines activity	<i>In vivo</i>	U937 human leukemia & ICR mice
MMHO-1	Inhibits angiogenesis	VEGF activity	<i>In vitro</i>	Bovine aortic endothelial cells
Antroquinonol	Induce apoptosis	Mitotic-catastrophe	<i>In vitro</i>	U937 & BxPC3 pancreatic cancer cell
Antroquinonol	Cell cycle arrest	AMPK and mTOR activity	<i>In vitro</i>	HCCs, HepG2, HepG2.2.15, Mahlavu, PLC/PRF/5, SK-Hept & Hep3B cancer cells
Antroquinonol	Induce cytotoxicity	Unknown	<i>In vitro</i>	MCF-7, MDA-MB-231, HepG2, Hep3B, Du-145 & LNCaP*
Antroquinonol	Cell cycle arrest	mTOR activity	<i>In vitro</i>	NSCLC human non-small lung cancer cell line
Antroquinonol	Inhibits proliferation	COX-2 and MDR1 activity	<i>In vitro</i>	HepG2 human liver cancer cell
Anticinate	Inhibits metastasis	Akt/mTOR activity	<i>In vitro</i>	MDA-MB-231 human breast cancer cell*
Methyl anticinate-A	Induce apoptosis	Caspase activity	<i>In vitro</i>	HepG2 and Hep3B human liver cancer cells
Methyl anticinate-A	Induce apoptosis	Caspase activity	<i>In vitro</i>	OEC-M1 and OC-2 human oral cancer cells

Table 1. Anti-cancer activity of *A. camphorata* and its components against various cancer models

## 2. *A. camphorata* regulates cell cycle progression

In mammalian cells, cell cycle progression is tightly coordinated by the cyclin-dependent protein kinases (Cdk1, Cdk2, Cdk4 and Cdk6), regulatory cyclin subunits (cyclin-A, cyclin-B, cyclin-Ds and cyclin-E) and their inhibitors including p21<sup>WAF1</sup> and p27<sup>KIP1</sup> (Athar et al., 2009). Cyclin-E/Cdk1 (Cdc2) complex is the key components of the cell cycle check point pathway that delays mitotic entry in response to stalled replication or DNA damage. In addition to Cdk1, cyclin-Ds/Cdk4/6, and cyclin-A/Cdk2 are also required for G1/S transition and progression through S phase, while cyclin-A,B/Cdk1 complex activates are required for entry into mitosis (Fig. 1). Although, cyclin-B/Cdk1 complex are maintained inactive during interphase through the phosphorylation of Cdk1 at Thr 14 and Tyr 15 residues, which are catalysed by Wee1 and Myt1 (Thomas et al., 2005). Cyclin-D1 is a rate limiting activator for the G1/S transition, another cell cycle check point. The G1/S transition requires the activation of the cyclinD/Cdk4/Cdk6 and cyclin-E/Cdk2 complexes, which in turn phosphorylates the retinoblastoma protein (Rb). The subsequent dissociation of E2Fs from Rb activates a series of target genes that are required for cell entering S phase (Athar et al., 2009). Rb was the first tumor suppressor gene, which is essentially hypophosphorylated when cells are in G0 and become progressively phosphorylated by G1 phase cyclin/Cdk complexes as cells enter G1, becoming hypophosphorylated on a larger number of serine and threonine residues as cells advance through the R point., Rb remains hypophosphorylated through the remainder of the cell cycle. The phosphorylate groups on Rb are removed by the protein phosphatase (PPI) as cells exit mitosis. Therefore, Rb plays a critical role in cell cycle progression as the molecular governor of the R-point transition (Mathews & Gerritsen, 2010). Besides, Cdk2 activation, entry into mitosis requires nuclear translocation of active Cdc2/cyclin-B1 complexes. Normally, most Cdc2/cyclin-B1 complexes accumulate in the nucleus during prophase while Cdc25C phosphatase activates them by dephosphorylating Cdc2 on both Thr14 and Tyr15 (Thomas et al., 2005).

Cell cycle kinases are frequently upregulated in human cancer due to the over expression of their cyclin partners or inactivation of the Cdks inhibitors. Indeed, deregulation of cyclin-D1-Rb axis is very common in human cancers as cyclin-D1 accumulation is found in various types of human malignancies including breast, skin, lung, liver etc., and affect cell cycle modulation perhaps its most extensively studied target (Athar et al., 2009). Number of researchers has been reported on the cancer preventive and therapeutic effects of *A. camphorata* in different *in vitro* and *in vivo* test models. As summarized in Table. 1, various parts including mycelium and fruiting body, extracts (ethanol, methanol and ethyl acetate extracts) and chemical ingredients such as polysaccharides, triterpinoids, sesquiterpine, steroids, phenol compounds, adenosine, cordycepin, ergosterol etc., possessed potent anticancer activity. Besides, breast cancer cells are highly resistant to chemotherapy, and there is still no effective cure for patients with advanced stages of the disease, specifically in cases of hormone-independent cancer (Rao et al., 2011). In addition, the cost of this therapy is significant, and therefore

The inhibition of breast cancer cell proliferation by *A. camphorata* was strongly associated with cell cycle arrest and/or induction of apoptosis. Exposure of human breast cancer cells (MCF-7) against *A. camphorata* caused significant arrest of cell progression (Fig. 2) in G1 phase (Yang et al., 2006). It was further confirmed by estrogen non-responsive human breast cancer cell line (MDA-MB-231) showed that approximately 70% of *A. camphorata* treated MDA-MB-231 cells were arrested in sub-G1 (Fig. 2) phase (Hseu et al., 2008). It has

been documented that *A. camphorata* also arrest androgen-responsive-LNCaP and androgen-independent PC-3 human prostate cancer cells in a similar G1/S (Fig. 2) phase arrest (Chen et al., 2007), but caused G0/G1 arrest in human hepatoma HepG2 cells (Song et al., 2005), whereas, human urinary bladder cancer (T24) cells were arrested in G2/M (Fig. 2) phase (Peng et al., 2007). The reasons as to why *A. camphorata* causes G1 arrest in breast cancer cells, but G2/M phase arrest in other cells are still unknown. Therefore, *A. camphorata* has been shown to modulate the major cell cycle mediators at lower microgram concentrations, arresting breast cancer cells at the G1/S phase of the cell cycle. The anti-proliferative activity of *A. camphorata* involves the induction of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> and down-regulation of cyclin-A/D1/E, cdc2, and CDK4 (Hseu et al., 2008).

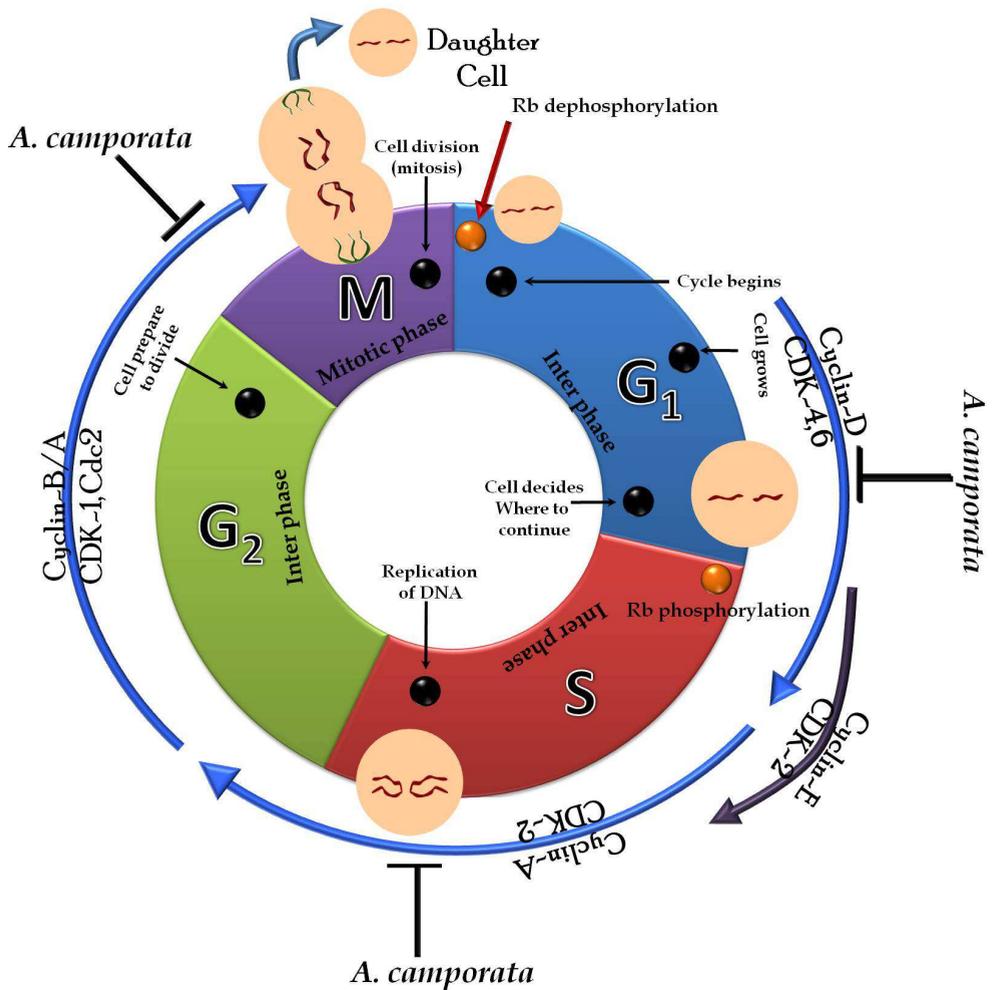


Fig. 2. Schematic diagram of *A. camphorata*-induced cell cycle arrest in various cancer cells

### 3. A. *camphorata* regulates apoptosis and cell survival

The major strategies of the underlying cause of cancer was attributed to accelerated or dysregulated proliferation leading to cellular expansion and accumulation of tissue mass. It is well understood that key regulators of the cell cycle are frequently altered in many tumor types, with a consequent impact on elements of proliferative control such as cell cycle checkpoints and the response to DNA damage. Therefore, modern chemotherapeutic approaches are designed to exploit such aberration to induce cytotoxicity and tumor regression or cytostasis to control tumor progression. Uncontrolled cell proliferation, however, is only part of the picture. Recent cancer research progress has broadened our understanding underlying etiology to encompass aberrant cellular survival, as a consequence of failing to appropriately induce apoptosis or cell death, which are major contributor to the transformed state. Apoptosis, also known as programmed cell death, is a well-regulated and ordered process that occurs both in development and in response to stress to help maintain tissue homeostasis. Environmental and physico-chemicals stimulates accumulation of mutations or carcinogens that critically alter cell proliferation, cell cycle regulation, cell-cell or cell-extra cellular matrix (ECM) interactions, which eventually leads malignancy. However, apoptosis induction helps to prevent malignancies via eliminating damaged cells (Leibowitz & Yu, 2010). Apoptosis occurred *via* multiple pathways, and the extrinsic death receptor-mediated and the intrinsic mitochondrial-mediated cell death pathway (Fig. 3) are the ones better characterized in molecular terms (Fulda & Debatin, 2006).

The role of mitochondria as principal crossroad of the apoptotic process and emerged since 1990's, when it was shown that mitochondria of apoptosing cell death (Ghibelli & Diederich, 2010). The intrinsic pathway is characterized by the rapid release of cytochrome *c* from the mitochondrial inner membrane space into the cytosol. This critical event is absolutely required for caspase-dependent cell death (Kasibhatla & Tseng, 2003). The term mitochondrial outer membrane permeabilization (MOMP) was coined (Green & Kroemer, 2004), which indicates release of inter-membrane proteins rather than ion passage. However, the topological features and size concerns questioned about cytochrome *c* release *via* phospholipids transfer protein (PTP). A channel linking the inter-membrane mitochondrial space to the cytosol was sought to explain release of cytochrome *c*. The release of cytochrome *c* also depended activation of pro-apoptotic proteins such as Bax/Bid, which was stimulated by physico-chemical-induced cell stress. During the stimulation, cytochrome *c* nucleates the assembly of a multi-protein complex, known as apoptosomes, functionally analog to the DISC, further recruits and activates the other upstream caspases, including caspase-9 and caspase-8. Caspase-8 and caspase-9 converge into proteolytic activation of caspase-3, results cells undergo execution phase of apoptosis and/or cell dismantling (Ghibelli & Diederich, 2010).

Number of investigations critically characterized the anticancer potential of *A. camphorata*, regarding to the potential cytotoxic effect of various cancer cells derived from different human origins including lung, liver, breast, prostate, colon and oral cells (Table. 1). The viability of these cancer cells was significantly decreased by *A. camphorata* treatment in a dose-dependent manner. However, different cancer cells responded to *A. camphorata* with different sensitivities. A crude aqueous extract obtained from the fermented culture broth of *A. camphorata* exerted a potent effect in reducing the viability of different human breast cancer cell lines, including estrogen-responsive MCF-7 and estrogen-nonresponsive MDA-

MB-231 cells with an IC<sub>50</sub> value of 57 and 136 µg/mL, respectively (Yang et al., 2006; Hseu et al., 2008; Yang et al., 2011). Meanwhile, the highest dose of *A. camphorata* (>100 µg/mL) was used to treat normal human endothelial cells for 24 hours, there was no cytotoxic effects were found. Furthermore, the chloroform extracts of fruiting bodies of *A. camphorata* significantly inhibited the growth of human breast cancer (MCF-7) cells with an IC<sub>50</sub> value of 65 µM (Rao et al., 2007). This indicated the differential cytotoxic effects of *A. camphorata* crude extract on different kinds of breast cancer cells, with no harmful effects on normal cells at higher concentrations (Hseu et al., 2002).

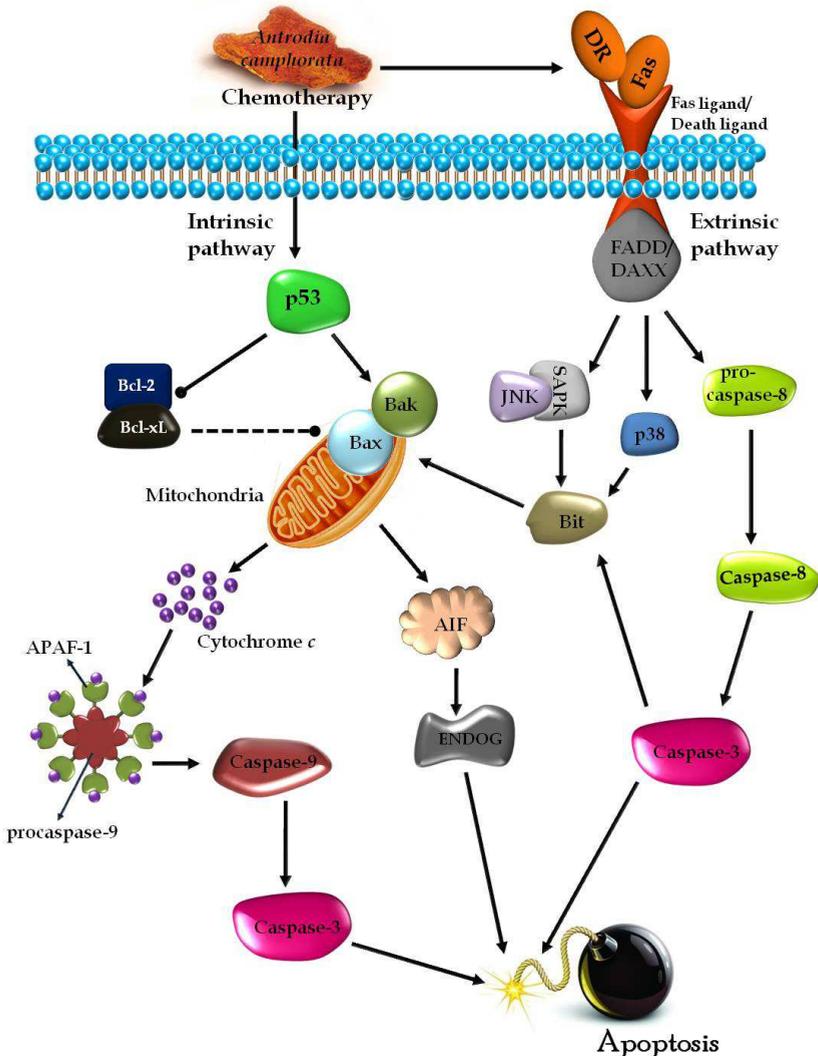


Fig. 3. Schematic representation of *A. camphorata*-induced apoptosis in *via* intrinsic and extrinsic pathways

In recent years much interest has been focused investigation onto the cancer preventive compositions of *A. camphorata* rather crude extract. There are two review articles available, thus, extensively summarized potent bioactive components, which are present in *A. camphorata* (Ao et al., 2009; Geethangili & Tzeng, 2009). It is believed that the anti-cancer and anti-metastasis properties of this mushroom are derived from its diversified chemical constituents, although it is comprised primarily of two types of compounds: polysaccharides and triterpenes (Shao et al., 2008). Yeh et al. demonstrated that five lanostanes (dehydroeburicoic acid, 15 $\alpha$ -acetyl dehydrosulphurenic acid, 24-triene-21-oic acid, dehydrosulphurenic acid and sulphurenic acid) and three ergostane-type triterpenes (zhankuic acid, zhankuic acid-A and zhankuic acid-C) isolated from fruiting bodies of *A. camphorata* exhibits in vitro cytotoxic effect to various cancer cell lines, including MDA-MB-231. Zhankuic acid and sulphurenic acid showed significant cytotoxic effect to the human breast cancer cells MDA-MB-231 and MCF-7 with an IC<sub>50</sub> value of 25.1 and 89.2; and 57.8 and 357.0  $\mu$ M, respectively. Indeed, sulphurenic acid is selectively cytotoxic to estrogen-nonresponsive MDA-MB-231 cells, and the only one compound that does not contain the diene structure with in the rings at position 7 and 9 and instead possesses a single double bond in the rings at the position 8 (Yeh et al., 2009). This result also provides another hallmark that cytotoxic effect of bioactive compounds also depends on structural activity relationship. Antroquinonol, an ubiquinone derivatives, was isolated from the solid state fermented mycelium of *A. camphorata* exhibits cytotoxic effect against MDA-MB-231 and MCF-7 human breast cancer cells with an IC<sub>50</sub> value of 2.64 and 2.1  $\mu$ M, respectively (Lee et al., 2007). A very similar result were obtained from an another pure compound antrocin, isolated from the fruiting bodies of *A. camphorata* showed the most anti-proliferative effect against MDA-MB-231 and MCF-7 cells (Rao et al., 2011). Notably, non-tumorigenic breast epithelial MCF-10A cells were not affected by antrocin treatment.

The morphological changes of human breast cancer (MCF-7) cells after *A. camphorata* treatment were further investigated to elucidate the underlying process of reduced viability treatment. The typical morphological characteristics for cell apoptosis, such as cell condensation, plasma membrane blabbing and formation of apoptotic bodies, were confirmed under phase contrast microscopy (Yang et al., 2006). The hallmark of cell apoptosis, DNA fragmentation was also demonstrated in *A. camphorata* treated human breast cancer cells including MCF-7 and MDA-MB-231. Caspase-3 activity in *A. camphorata*-treated MCF-7 breast cancer cells was shown to be increased, which further confirmed that *A. camphorata* could induce apoptosis in human breast cancer cells (Yang et al., 2006). An addition to *A. camphorata* crude extract, antrocin, a pure compound isolated from the fruiting bodies of *A. camphorata* significantly induced apoptosis in MDA-MB-231 breast cancer cells through the activation of caspase-3 (Rao et al., 2011). *In vivo* study, we showed that the tumor formation in BALB/*c-nu* mice with the implantation of MDA-MB-231 cells could be inhibited by the administration of the aqueous fermented culture broth of *A. camphorata*. The initial tumor development was dose-dependently inhibited by *A. camphorata*. The significant suppression of tumor development was directly demonstrating the *in vivo* anti-tumor effect of *A. camphorata* (Hseu et al., 2008). Comparably there was no cytotoxic effect was observed in control mice that received *A. camphorata* alone.

The mechanism of apoptosis induced by *A. camphorata* with various cancer cell lines were extensively studied (Table. 1) and demonstrated the relevant pathways of apoptosis (Fig. 3). There is an increase of the Bax/Bcl-2 ratio associated with the apoptosis induced by *A. camphorata* treatment in MCF-7 and MDA-MB-231 cells (Fig. 3). MCF-7 cells exposed to *A.*

*camphorata* significantly enhanced Bax protein expression, whereas, Bcl-2 the anti-apoptotic protein was not affected (Yang et al., 2006). Rao et al. studied that antrocin, a pure compound isolated from the fruiting bodies of *A. camphorata* markedly augmented Bax:Bcl-2/Bcl-xL ratio in MDA-MB-231 cells (Rao et al., 2011). In an *in vivo* model, Bcl-2-positive cells were observed in MDA-MB-231 cells implanted mice tumor tissue. Furthermore, TUNEL assay showed that *A. camphorata*-induced Bcl-2 inhibition was directly proportional to apoptotic cells in mice tumor tissue (Hseu et al., 2008). This study also confirmed that *A. camphorata*-induced tumor suppression was mediated by cell-cycle arrest, as evidenced by reduction of cyclin-D and PCNA protein levels in mice tumor tissues. The down-regulation of Bcl-2 expression is known to be involved in the release of cytochrome *c* from mitochondria from the intrinsic pathway (Fig. 2). Further we investigated the *A. camphorata*-induced mitochondria membrane permeability and cytochrome *c* release in MCF-7 cells. Cells treated with *A. camphorata* significantly increased cytochrome *c* accumulation in cytoplasm, which supports *A. camphorata*-induced mitochondrial membrane potential. This data also provided another possible mechanism that *A. camphorata*-induced apoptosis was mediated by mitochondrial membrane potential followed by cytochrome *c* release in human breast cancer cells (Yang et al., 2006).

Another important mediator of apoptosis in immune cells is the Fas receptor/ligand signaling system. The critical elements of the Fas pathway that link receptor-ligand interaction and down-stream activation of caspases, including caspase-3, have been identified (Hung et al., 2010). Recent studies also indicate that widely used chemotherapeutic agents induce apoptosis in susceptible cells. Thereby, the chemotherapeutic agents required an alternative mediator. Recent studies revealed that Fas/Fas ligand (FasL) or death ligand/death receptor (DR) can activate the downstream extrinsic apoptotic pathway (Fig. 2). Gene expressions of both Fas/FasL were induced in human hepatoma HepG2 cells by treatment with methanolic extract of mycelium of *A. camphorata*. However, *A. camphorata* treatment dose-dependently inhibits death receptors (DR-4 and DR-5) and TNF- $\alpha$  receptors (TNFR-I and TNFR-II) in HepG2 cells.

Indeed, these results well demonstrate that *A. camphorata* induced apoptosis possibly by involving up-regulation of Fas expression, which promotes the ligation of Fas and FasL and then passes the death message to cytosolic messengers. As a result, procaspase-8 is activated to caspase-8, which triggers the caspase activation cascade. In addition, this study also demonstrates that *A. camphorata*-induced apoptosis was mediated by mitochondrial-independent pathway (Song, 2005).

#### **4. *A. camphorata* up-regulates tumour-suppressor genes**

The p53 tumor suppressor gene encodes a multifunctional protein involved in the comprehensive control of cellular responses to genotoxic stress. p53 mediated tumor suppressor effects are mediated by a variety of mechanisms including cell cycle arrest, apoptosis and cellular senescence that prevent cells with damaged DNA to pass on their genome to progeny. Recently much attention has been focused towards p53, because it is one of the main effectors of cell cycle check point. However, the precise molecular mechanisms of its action are still controversial. Several reports indicate that p53 directly arrest cells in G1 phase in response to DNA damage, thus preventing DNA synthesis from damaged templates (Wahl et al., 1997). Apart from this p53 is involved in regulating the cell cycle at transition of G1/S and G2/M and with in S phase (Talos & Moll, 2010). Evidence for

a possible role of p53 in M phase came from observations that p53 contributes to the control of centrosome duplication and to the prevention of DNA replication is impaired by spindle inhibitors (Talos and Moll, 2010).

In recent reports indicate that both estrogen receptor positive (MCF-7) and triple negative (MDA-MB-231) breast cancer cells were exposed to synthetic or natural derived anti-cancer drugs remarkable arrest cell cycle via accumulation in G2/M phase through the inhibition of Akt activity and p53-independent or p53-dependent activation of p53 inducible proteins such as p21/p53R2 /CDKN1A and GADD45A (De Santi et al., 2011; Hahm et al., 2011; Hsieh et al., 2011). It is noteworthy that *A. camphorata* significantly up-regulates p53 tumor suppressor gene in human colorectal carcinoma cells (Lien et al., 2009), and human prostate cancer cell lines (Chen et al., 2007). However, the potential up-regulation of p53 tumor suppressor gene product by *A. camphorata* was yet to be illustrated in human breast cancer cell lines. Our current work is fascinating *A. camphorata* induced up-regulation of p53 in triple negative MDA-MB-231 cells. We believe coming future this result may give vital evidence that anti-tumor activity of *A. camphorata* through the up-regulation of p53 tumor suppressor gene.

### **5. A. camphorata down-regulates invasion and metastasis**

Metastasis is characterized that the multistep processes by which cancer spreads from the place at which it first arose as a primary tumor to distant location and establish itself in a new site in the body through blood stream or lymphatic system. Metastasis depends on the cancer cells acquiring two separate abilities increased motility and invasiveness cells that metastasize are basically identical to the original tumor. For example if cancer arises in the breast and metastasizes to the lung, the cancer cells in lung are similar breast cancer cells. Normally our body has many safeguards to prevent cells from adverse cancerous effects. However, many cancer cells itself have the ability to overcome these safeguards. In recent years much research has been focused on to understanding how cancer cells are mutated to circumvent the body's defenses and freely travel to another location. In normal tissue, cells adhere both to one another and a mesh of proteins are filled the space between them, this outer membrane proteins are known as extracellular matrix. The connection between cells and extracellular matrix is particularly characterized that from skin, mouth, lung, stomach and other organs. During invasion, cells spreads, it must break away not only from the cells around it, but also from the extracellular matrix. Cells are tightly bonded with cell-to-cell adhesion molecules. These adhesion molecules also allow interactions between numerous proteins on the cells surface. In cancerous cells, the adhesion molecules seem to be missing or compromised.

Cadherins, a family of Ca<sup>2+</sup>-dependent intracellular adhesion protein molecules, which playing vital role for connecting two individual cells. Cadherins also regulates cells morphology, motility and hence tumor invasiveness. The cadherin molecules have three major regions. There is an extracellular region that mediates specific adherins, a transmembrane domain that spans the cell membrane, and a cytoplasmic domain that extends into the cell. The normal pattern of E-cadherin,  $\alpha$ -,  $\beta$ -,  $\gamma$ - and p20-catenin are strong membraneous staining with localization at the intracellular border of luminal cells. Abnormal (absent, reduced or localized to cell compartments other than cell membrane) expression of E-cadherin and catenin has been reported in various human cancers and has been associated with tumor progression. The degradation of  $\beta$ -catenin involves its

phosphorylation through complex formation with tumor suppressor gene products such as adenomatous polyposis coli (APC), glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) and axin (Davidson et al., 2000). In tumor cells, E-cadherin is either partly or entirely missing. This allows tumor cells to detach from each other, and from the matrix which holds everything in place. Recent clinical studies revealed that E-cadherin is important to regulate metastasis. E-cadherin-mediated cell-cell adhesion is associated with the progression of many carcinomas, including breast, bladder and squamous head and neck carcinomas (Davidson et al., 2000). Abnormal expression of E-cadherin and catenin is very common in lobular than ductal carcinomas. However, its expression appears not to be involved into the early stage of neoplasia but to correlate with high grade invasive ductal carcinoma (Nakopoulou et al., 2002). Reduced expression of E-cadherin is seen in about 50% of ductal carcinomas of the breast and is associated with high histological grade, nodal metastases and poor prognosis. Addition to cadherins, a number of proteolytic enzymes contribute to the degradation of environmental barriers, such as the extracellular matrix and the basement membrane. Thus, degradation of the extracellular matrix and components of the basement membrane, mediated by the concerted action of proteinases, such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), play a critical role in tumor invasion and metastasis (Westermarck & Kahari, 1999).

Increasing expression of MMP-2, MMP-9 and angiogenic cytokine vascular endothelial growth factor (VEGF) in human breast cancer cell lines including estrogen receptor positive (MCF-7), triple negative (MDA-MB-231) and ductal epithelial tumor (T47D) cells has been suggested to be associated with the highly metastatic potential of breast cancer (Shibata et al., 2002; H.S. Lee et al., 2008). The promoter region of MMP-2 contains various cis-acting elements, including potential binding sites for the transcription factors nuclear factor-kappa B (NF- $\kappa$ B), activator protein-1 (AP-1), and stimulatory protein-1 (SP-1) (Lin et al., 2010). NF- $\kappa$ B is critically involved in tumor progression through transcriptional regulation of invasion related factors, such as MMP-2/MM-9, uPA and VEGF (Shibata et al., 2002; Yang et al., 2011). In addition, the involvement of mitogen-activated protein kinase (MAPK) pathways in NF- $\kappa$ B activation has been demonstrated to play an important role in tumor metastasis (Yang et al., 2011). Therefore, the inhibition of MMPs- and/or uPA-mediated migration or invasion could be a potential treatment for preventing or inhibiting cancer metastasis.

Once breast cancer has spread beyond the breast and under arm lymph nodes, it is considered a "systemic" disease, meaning that it is necessary to treat the whole body rather than just one particular spot. If breast cancer cells has traveled through the blood stream or lymphatic system, there are likely to be breast cancer cells in many different parts of the body, even scans only shows few spots. Therefore, the treatment that reaches all parts of the body. Chemotherapy or hormonal therapy, are more suggestible treatment are used to treat metastatic breast cancer instead of treatments that just treat one part of the body, just as surgery. In general, a women might be treated with a hormonal therapy if she has a hormone responsive (estrogen or progesterone receptor positive) tumor, whereas, tumor that are not responsive to hormonal therapy, further choice is chemotherapy. There are many different types of chemotherapy that are used for breast cancer. In recent years, there has been much interest in developing new types of medicines that kill breast cancer cells in new and different way. Some of these drugs are only work against a specific type of breast cancer. To overcome this problem, current drug discovery system more fascinating a target protein or signaling cascades rather than specific type of breast cancer cells.

It was well demonstrated that metastasis is responsible for the majority of failures in cancer treatment, and is the major cause of death from cancer. Therefore, chemotherapy is suggested to prevent local recurrence of the primary tumor and the spread of tumor cells (Weng & Yen, 2010). However, commercially available synthetic chemotherapeutic agents have severe side effects. Recent studies demonstrated that phytochemicals derived from plant source potentially prevents cancer metastasis (Sliva, 2008). As I mentioned that *A. camphorata* has been used in traditional Chinese medicine for various ills, including cancer. The mechanisms of action of *A. camphorata* against cancer cells includes inhibition of cell proliferation, induction of apoptosis and suppression of the motility of highly invasive breast cancer cells were intensively studied (Yang et al., 2006; Hseu et al., 2008). Although there are different compounds with various pharmacological activities were extracted from mycelia, fruiting bodies, spores and fermented culture broth of *A. camphorata*. The anticancer and anti-metastatic activities of this medicinal mushroom were primarily relied on its polysaccharide, benzenoids, lignans, diterpenes, triterpinoid and steroid components (Ao et al., 2009; Geethangili & Tzeng, 2011).

Our recent study was clearly demonstrated that the anti-invasive and anti-metastatic effects of *A. camphorata* against highly metastatic human breast cancer cells (MDA-MB-231) is due to the inhibition of invasion and metastasis regulatory proteins such as MMP-2, MMP-9, uPA, uPA receptor and VEGF through the down-regulation of MAPK/NF- $\kappa$ B signaling pathway (Yang et al., 2011). *A. camphorata* inhibits invasion and metastasis of breast cancer cells not only through the suppression of MMPs/uPA, it also enhance endogenous MMPs/uPA inhibitors, TIMP-1, TIMP-2 and PAI respectively. It is also well understood that *A. camphorata* potentially modulates MAPKs cascades (Geethangili & Tzeng, 2009). Our investigation also revealed that the inhibition of MMPs/uPA due to the down-regulation of MAPK cascades such as ERK1/2, JNK and p38. Further we observed the major MMP's transcription factor NF- $\kappa$ B also significantly inhibited by *A. camphorata* treatment in MDA-MB-231 human breast cancer cell (Yang et al., 2011).

Moreover, there is substantial evidence on the inhibition of MMP-9/MMP-2 and suppression of invasiveness and metastases of cancer cells using various chemopreventive or chemotherapeutic agents (Ho et al., 2002; Abiru et al., 2002). Based on the observation, *A. camphorata* eventually decreased the activity or protein levels of tumor metastasis-related proteins, including MMP-9, MMP-2, uPA, and uPAR, and increase the expression of their endogenous inhibitors, TIMP-1, TIMP-2, and PAI-1, in MDA-MB-231 cells. Therefore, *A. camphorata* could be a potential agent for the prevention of breast cancer metastasis.

## **6. A. camphorata down-regulates tumour angiogenesis**

Angiogenesis is the development of a new blood supply from an existing vasculature. Normal cells can stimulate new blood vessels to grow. This happens to repair damaged tissue when wounds are healing. Therefore, normal cells have genes that can produce proteins known as angiogenic factors, which switch blood vessels growth on. However, cells also have genes that produce certain molecules called anti-angiogenic factors, which slow down blood vessel growth (Papetti & Herman, 2002).

Accumulating evidences indicate that progressive tumor growth is dependent on angiogenesis. It also plays an important role in the growth and spread of cancer. New blood vessels "feed" the cancer cells with oxygen and nutrients, allowing these cells to grow, invade nearby tissue, spread to other parts of the body, and form new colonies of cancer

cells (Maliwal et al., 2009). Every cancer begins its existence as a tiny cluster of abnormal tumor cells growing in an organ. Without its own blood supply to bring in oxygen and nutrients, the tumor cannot grow larger than 1-2 millimeters in diameter (about the size of a small pea). While this early stage of tumor growth can last for month or even years, eventually a few cancer cells gain the ability to produce angiogenic growth factors. These growth factors are released by the tumor into nearby tissues, and stimulate new blood vessels to sprout vigorously from existing healthy blood vessels toward and into the tumor. In addition, increased angiogenesis has also been observed in preneoplastic conditions (Sharma et al., 2001), indicating that it plays a key role in the early processes of carcinogenesis.

Various angiogenic regulators have been identified since the introduction of angiogenesis in the scientific community. As we mentioned above that the stability of vasculature is highly regulated by the homeostasis between angiogenic stimulators and inhibitors. The best characterized angiogenic stimulators including, angiopoietin, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), endothelial growth factor (EGE) and hepatocyte growth factor (HGF) (Liekens et al., 2001; Takeya et al., 2008). Number of research is going on onto anti-angiogenic therapy. The research has found that the amounts of these angiogenic factors are expressed very high at the outer edge of tumor. Anti-angiogenic drugs may stop a cancer from growing into surrounding tissue or spreading. They will probably not be able to get rid of a cancer, but may be able to halt new blood vessel growth and starve a tumor by cutting off its blood supply. There are more than three hundred angiogenesis inhibitor molecules have been discovered so far: Some angiogenesis inhibitors are naturally present in the human body (endogenous angiogenic inhibitors) results healthy tissues appear to resist cancer growth by containing these antiangiogenic compounds. The endogenous angiogenic inhibitors are classified into five major groups; 1) endothelial cell specific inhibitors, 2) avascular tissue-derived inhibitors, 3) anti-angiogenic cytokines, 4) angiogenic factor antagonists and 5) other inhibitors (Cao, 2001; Grant & Kullar, 2005). Other angiogenesis inhibitors occur naturally in substances found in green tea, soy beans, fungi, mushrooms, tree bark, shark tissues, snake venom and many other plants and animals. Still other angiogenesis inhibitors have been manufactured synthetically in the laboratory.

Conventional chemotherapy preferentially targets rapidly dividing cancer cells. However, certain normally dividing cells (hair cells, intestinal cells, mucous membranes, bone marrow cells) are also destroyed, which causes the well-known severe chemotherapy side effects of hair loss, diarrhea, mouth ulcer, infection, and low blood counts. Some chemotherapy regimens work very well at treating cancers that are diagnosed early. Most of the anti-angiogenic therapies targets only growing new blood vessel (endothelial) cells. Since blood vessels do not grow in normal, healthy tissues, the side effects of anti-angiogenic therapy are concentrated primarily at the cancer site. Most anti-angiogenic drugs do not kill cancer cells directly and are therefore better tolerated compared to chemotherapy, with fewer and less severe side effects. To keep cancers from re-growing, it is possible that some patients may need to take anti-angiogenic drugs as a chronic therapy, although this hypothesis is still being tested in clinical studies. In response to targeted affects anti-angiogenic agents are classified into three major class; they are 1) agent that block new blood vessel from sprouting (true angiogenic inhibitors), 2) assault a tumor's established blood supply (vascular

targeting agents) and 3) attack both the cancer cells as well as blood vessel cells (the double-barreled approach). Recent clinical studies in different cancer types have shown that anti-angiogenic therapy generally works best when used in combination with cytotoxic chemotherapy or radiation. Most experts believe that such combinations will ultimately provide cancer patients with the greatest benefit.

A recent study has shown that ethyl acetate extracts of fruiting bodies of *A. camphorata* not only suppressed tumor growth in human liver cancer cell PLC/PRF/5 xenografted male BALB/cA-*nu* nude mice, but also inhibits tumor angiogenesis (Hsu et al., 2007). It is now known that a decrease in tumor size is often associated with inhibited microvessel formation in the tumor. This study also confirmed that PLC/PRF/5 cells implanted mice greatly induced angiogenesis and the hemoglobin levels (6.4-fold). The amount of hemoglobin level in tumor tissue is considered as a blood vasculature in tumor tissue. However, mice were pretreated with *A. camphorata* significantly inhibits PLC/PRF/5 cells-induced angiogenesis and hemoglobin level in mice tumor tissue (Hsu et al., 2007).

Subsequently, polysaccharides were isolated from the mycelium or fermented culture broth of *A. camphorata* showed potent anti-tumor activity in several in vitro and in vivo models (Liu et al., 2004). Tumor growth and metastasis is angiogenesis dependent. Several lines of direct evidence have shown that angiogenesis is essential for tumor growth and metastasis (Weng & Yen, 2010). The *ex vivo* check chorioallatonic membrane (CAM) assay is commonly employed to examine the anti-angiogenic activity of samples. Polysaccharides with different molecular weight, isolated from *A. camphorata* were tested for its anti-angiogenic properties using CAM assay. The microvasculature was markedly reduced after treatment with polysaccharides (Yang et al., 2009). In addition to CAM assay, Tube forming assays measure a complex series of events involving changes in endothelial cell morphology and migration, leading to the formation of a complex interconnecting network of capillary tubes with identifiable lumens (branching morphogenesis) (Cavell et al., 2011). Polysaccharides from *A. camphorata* also significantly inhibit matrigel-dependent capillary tube formation in human umbilical vein endothelial (HUVEC) cell and bovine aortic endothelial cells (Yang et al., 2009; Chen et al., 2005; Cheng et al., 2005).

Angiogenesis requires endothelial proliferation, migration, and tube formation. Cancer cells are able to produce large amounts of several angiogenic factors including VEGF, EGF, FGF (Liekens et al., 2001). Therefore, VEGF is considered as an important biomarker of angiogenesis. Over expression of VEGF in tumors increases tumor vascularization and growth, while capturing VEGF or blocking its signaling receptor, VEGFR-2, by VEGF receptor tyrosine kinase inhibition, antisense oligonucleotides, vaccination, or neutralizing antibodies reduces tumor angiogenesis and growth. Besides, VEGF induces cyclin D1 expression, which serves as a cell cycle regulatory switch in actively proliferating cells, through PLCg-PKC-MAP kinase pathways. Further to confirm the anti-angiogenic activity of *A. camphorata* the inhibition of VEGF-R tyrosine kinase phosphorylation was monitored. Similarly, polysaccharides from *A. camphorata* have been shown to suppressed VEGFR-2 tyrosine kinase phosphorylation in Tyr1054/1059 residual position. This study also revealed *A. camphorata* significantly inhibits endothelial cell proliferation as evidenced by the reduction of cyclin-D1 protein expression, which is a marker of cell cycle check point (Cheng et al., 2005). These results indicate that *A. camphorata* might be a potent inhibitor of angiogenesis and subsequent tumor promotion.

## 7. A. *camphorata* down-regulates NF- $\kappa$ B/AP-1 signalling pathway

### 7.1 Inhibits NF- $\kappa$ B activation

NF- $\kappa$ B (Nuclear Factor-KappaB) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. NF- $\kappa$ B dimers are sequestered in the cytosol of un-stimulated cells *via* non-covalent interactions with a class of inhibitor proteins, called I- $\kappa$ Bs. Signals that induce NF- $\kappa$ B activity cause the phosphorylation of I- $\kappa$ Bs, their dissociation and subsequent degradation, thereby allowing activation of the NF- $\kappa$ B complex. The degradation of I- $\kappa$ B proteins that permits NF- $\kappa$ B molecules to move into the nucleus is also carried out by the proteasome but only after prior phosphorylation of I- $\kappa$ B by the IKKs. NF- $\kappa$ B can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF- $\alpha$  or IL-1, growth factors, lymphokines, and by other physiological and non physiological stimuli (Li & Verma, 2002; Verma, 2004).

The Rel/NF- $\kappa$ B family of transcription factors are involved mainly in stress-induced, immune, and inflammatory responses. In addition, these molecules play important roles during the development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures (Matsumori, 2004). Moreover, NF- $\kappa$ B family members have been implicated in neoplastic progression and the formation of neuronal synapses (Matsumori, 2004). NF- $\kappa$ B is also an important regulator in cell fate decisions, such as programmed cell death and proliferation control, and is critical in tumorigenesis (Thu & Richmond, 2010). An another study showed that angiocidin, which shown anti-tumor activity by blocking angiogenesis in various cancer cells through the suppression of NF- $\kappa$ B. However, in MDA-MB-231 cells, angiocidin significantly activate NF- $\kappa$ B and the *de novo* up-regulation of many downstream genes transcribed by NF- $\kappa$ B, including cytokines, inflammatory mediators and the cell cycle inhibitor p21(waf1) (Godek et al., 2011).

The molecular identification of its p50 subunit (v-REL) as a member of the reticuloendotheliosis (REL) family of viruses that provided the first evidence that NF- $\kappa$ B is linked to cancer (Prasad et al., 2010). Although the transforming ability of the v-REL oncoprotein was established many years ago, recent evidence suggests other human NF- $\kappa$ B family members may be important in oncogenesis (Dolcet et al., 2005). NF- $\kappa$ B DNA binding activity is constitutively increased in many lymphoid and epithelial tumors. The RAS, BCR-ABL, and HER2 oncogenes and transforming viruses can activate NF- $\kappa$ B. Furthermore, several genes thought to be essential to the cancer phenotype those controlling angiogenesis, invasion, proliferation, and metastasis, contain  $\kappa$ B binding sites.

Research over the past decade has revealed that NF- $\kappa$ B is an inducible transcription factor for genes involved in cell survival, cell adhesion, inflammation, differentiation and growth. Many of the target genes that are activated are critical to the establishment of early and late stages of aggressive cancers such as expression of cyclin D1, apoptosis suppressor proteins such as Bcl-2 and Bcl-XL and those required for metastasis and angiogenesis such as MMPs and VEGF (Dorai & Aggarwal, 2004). Higher concentration of serum VEGF has been shown to associate with a poorer prognosis in patients with breast cancer. On the other hand, constitutive expression of a transcription factor, NF- $\kappa$ B was correlated with progression and metastasis in a number of human breast cancers, suggesting a possible regulation of VEGF expression by NF- $\kappa$ B. Shibata et al. analyzed the expression of VEGF and constitutive NF- $\kappa$ B activity in three breast cancer cell lines, MCF-7, T47D, and MDA-MB-231. The basal levels of VEGF mRNA expression correlated with those of nuclear NF- $\kappa$ B activity in these cell lines. The highest NF- $\kappa$ B activity in MDA-MB-231 cells was associated with the highest expression

of VEGF mRNA, while the activity and the mRNA levels were moderate in MCF cells and the lowest in T47D cells (Shibata et al., 2002). A similar study showed that the triple negative breast cancers (MDA-MB231 and MDA-MB-468) or MCF-7 and T47D implanted mice expressed higher VEGF and NF- $\kappa$ B activation in their tumor tissues (Chougule et al., 2011; Antoon et al., 2011). Recently, Ambs and Glynn revived that inducible nitric oxide synthase (iNOS) has been observed in many types of human tumors. In breast cancer, increased iNOS is associated with markers of poor outcome and decreased survival. iNOS induction will trigger the release of variable amounts of NO into the tumor microenvironment and can activate oncogenic pathways, including the Akt, epidermal growth factor receptor and c-Myc signaling pathways, and stimulate tumor microvascularization. More recent findings suggest that NO induces stem cell-like tumor characteristics in breast cancer. This review, also pointed that NF- $\kappa$ B is the key transcription factor which playing major role for the production of NO *via* iNOS expression in various breast cancer cell lines (Ambs & Glynn, 2011). The over expression of metallothionein-2A (MT-2A) is frequently observed in invasive human breast tumors and has been linked with more aggressive breast cancers. MT-2A over expression led to the induction of MDA-MB-231 breast cancer cell migratory and invasive abilities. Concomitantly, they observed the expression of matrix metalloproteinase-9 (MMP-9) and the transcriptional activity of AP-1 and NF- $\kappa$ B were upregulated by MT-2A overexpression in MDA-MB-231 cells (Kim et al., 2011).

### 7.2 Inhibits AP-1 activation

Activated protein-1 (AP-1) is another transcription factor that regulates the expression of several genes that are involved in cell differentiation and proliferation. Functional activation of the AP-1 transcription complex is implicated in tumor promotion as well as malignant transformation. This complex consists of either homo or heterodimers of the members of the JUN and FOS family of proteins (Surh, 2003). This AP-1 mediated transcription of several target genes can also be activated by a complex network of signaling pathways that involves external signals such as growth factors, mitogen activated protein kinases (MAPK), extracellular-signal regulated protein kinases (ERK) and JUN-terminal kinases (JNK). Some of the target genes that are activated by AP-1 transcription complex mirror those activated by NF- $\kappa$ B and include Cyclin D1, bcl-2, bcl-XL, VEGF, MMP and urokinase plasminogen activator (uPA) (Dorai & Aggarwal, 2004). Expression of genes such as MMP and uPA especially promotes angiogenesis and invasive growth of cancer cells. Most importantly, AP-1 can also promote the transition of tumor cells from an epithelial to mesenchymal morphology which is one of the early steps in tumor metastasis. These oncogenic properties of AP-1 are primarily dictated by the dimer composition of the AP-1 family proteins and their post-transcriptional and translational modifications.

### 7.3 Inhibits COX-2 activity

Several preclinical studies indicated the importance of regulation of cyclooxygenase-2 (COX-2) expression in the prevention and the treatment of several malignancies. This enzyme is overexpressed in practically every pre-malignant and malignant condition involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck and esophagus (Aggarwal et al., 2006). Overexpression of this enzyme is a consequence of deregulation of transcriptional and post-transcriptional control. Depending upon the

stimulus and the cell type, different transcription factors including AP-1, NF-IL-6, NF- $\kappa$ B can stimulate COX-2 transcription (Surh, 2003). Wild type p53 protein expression can suppress COX-2 transcription while the mutant p53 protein can not. Consistent with this observation, increased COX-2 levels are seen in several epithelial cancers that express mutant p53. Taken together, these findings suggest that the balance between the activation of the oncogenes and the inactivation of the tumor suppressor genes and expression of several pro-inflammatory cytokines can modulate the expression of COX-2 in tumors. Complicating matters further is the fact that conventional cancer therapies such as radiation and chemotherapy can induce COX-2 and prostaglandin biosynthesis. Thus, inhibition of this enhanced COX-2 activity in tumors clearly has a therapeutic potential.

Accumulating evidence to implicate COX-2 function in breast cancer tumorigenesis. Soslowe et al. examined that 56% of infiltrating mammary carcinomas and intraductal carcinomas expressed significant levels of COX-2, while benign breast tissue at least 1 cm from a malignant lesion did not express COX-2 (Soslow et al., 2000). In a murine model of metastatic breast cancer, PGE<sub>2</sub> levels are positively correlated with increased tumorigenic and metastatic potential (Kundu et al., 2001). Perhaps the most convincing evidence that COX-2 causes breast cancer in animals comes from transgenic mice in which COX-2 was overexpressed in mammary tissue by using the mouse mammary tumor virus (MMTV) long-terminal repeat promoter. More than 85% of these mice developed tumors, indicating that COX-2 overexpression alone is sufficient to cause breast tumors (Liu et al., 2001). Ristimaki et al. analyzed the expression of COX-2 protein by immunohistochemistry in tissue array specimens of 1576 invasive breast cancers. Moderate to strong expression of COX-2 protein was observed in 37.4% of the tumors, and it was associated with unfavorable distant disease-free survival. Elevated COX-2 expression was associated with a large tumor size, a high histological grade, a negative hormone receptor status, a high proliferation rate (identified by Ki-67), high p53 expression, and the presence of HER-2 oncogene amplification, along with axillary node metastases and a ductal type of histology (Ristimaki et al., 2002). These results indicate that elevated COX-2 expression is more common in breast cancers with poor prognostic characteristics and is associated with an unfavorable outcome. Therefore the breast cancer treatment also targeted inhibition of COX-2 activity.

*A. camphorata* extract and its bioactive compounds, polysaccharides and triterpenoids, have been shown to have anti-proliferative, anti-invasive or anti-metastatic activities were observed in various cell lines or animal models. These investigations also revealed the *A. camphorata* regulation on signaling pathways or transcription factors, especially NF- $\kappa$ B involved in the effects of anti-angiogenesis, anti-adhesion and anti-invasion (Fig. 3). Moreover, the mechanism induced by *A. camphorata* with various cancer cell lines was well understood. We also contributed to understand the mechanism involved in breast cancer cells. Triple negative human breast cancer cells, MDA-MB-231 were treated with fermented culture broth of *A. camphorata* induced apoptosis followed by the regulation of Bax:Bcl-2 ratio. The induction of apoptosis was directly correlated with down-regulation of COX-2 expression in MDA-MB-231 cells (Hseu et al., 2007). This data supports *A. camphorata*-induced apoptosis might be associated with the inhibition of COX-2 activity. Very recently, we also observed that the fermented culture broth extracts of *A. camphorata* inhibit invasive behavior of breast cancer MDA-MB-231 cells through suppressing degradation of ECM, down-regulating the expression of MMPs, including MMP-9 and MMP-2; uPA and uPAR expression. This study also provided positive evidence that the inhibition of MMP-2, MMP-

9, uPA and uPAR in MDA-MB-231 cells by *A. camphorata* is through the down-regulation of MAP kinase cascades, including ERK1/2, JNK1/2 and p38. In addition, we also found that the inhibition of MAPKs further suppressed NF- $\kappa$ B nuclear translocation (Yang et al., 2011). Similar NF- $\kappa$ B, AP-1/MAPK and COX-2 inhibitory activity of *A. camphorata* was observed in various cancers and activated cell lines as summarized in (Geethangili & Tzeng, 2009; Ao et al., 2009). However, still certain molecular mechanisms are yet to be understood, especially the involvement *A. camphorata* in AP-1 transcriptional activation of inhibition in human breast cancer cells.

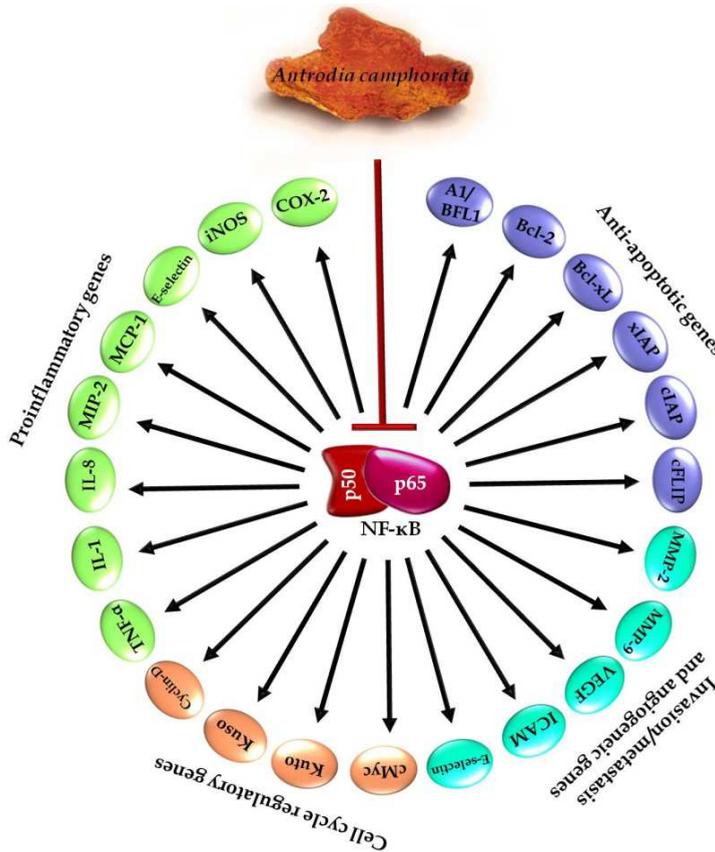


Fig. 4. NF- $\kappa$ B mediated anti-cancer activity of *A. camphorata*

## 8. Future perspectives

In past two decades *A. camphorata* received pioneering interest for their pharmacological interventions. It is obvious that *A. camphorata* exhibits anti-cancer activity against human breast cancer cell lines, including estrogen receptor-positive (MCF) and triple-negative (estrogen, progesterone and Her-2) MDA-MB-231 cell lines *in vitro* and *in vivo*. The pronounced anti-cancer activity was highly connected with its anti-metastasis, anti-

angiogenic, and the inhibition of cell cycle progression; and the induction of apoptosis in both intrinsic and extrinsic pathways. Besides, *A. camphorata* possessed various chemical components such as polysaccharides, triterpenes, diterpenes, benzoinoids and steroids may be the bioactive compounds responsible for the observed anticancer activity against human breast cancer cells. Still, there are number compounds which presented *A. camphorata* are warranted to extensive study. The presented evidences are confirmed that *A. camphorata* as a potential candidate for breast cancer chemoprevention. However, the chemopreventive agents can be used not just to prevent cancer but also treat cancer. Since, the pharmacological safety, most chemopreventive agents to enhance the effect at lower dose and thus can minimize chemotherapy-induced toxicity to non-cancer cells. It was cleared that *A. camphorata* failed to induce cytotoxic effect against non-cancer cell lines. And the notable point is certain human breast cancer cells are resistance to hormonal therapy or chemotherapy. Thus, agents that can suppress multiple pathways have great potential for the treatment of human breast cancer. *A. camphorata* achieved the target that it can inhibit cancer development in multiple signaling pathways.

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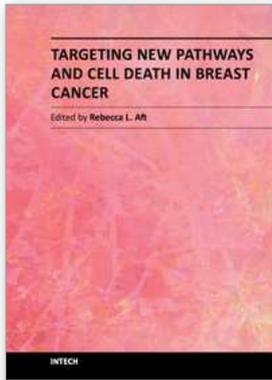
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