Medicinal and Aromatic Plant Science and Biotechnology ©2012 Global Science Books

Aloe Anthraquinones against Cancer

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ABSTRACT

Aloe has long been used in folk medicine for its curative and therapeutic properties, and two main classes of active compounds have been identified, namely anthraquinones and some characteristic β-polysaccharides. Among anthraquinones, aloe-emodin is reported to show the most interesting anticancer properties. This compound has been successfully tested against neuroectodermal cancer, leukemia, Merkel cell carcinoma and lung squamous cell carcinoma. Besides the effect on antioxidant enzymes is documented, several authors have identified the induction of cell apoptosis as the main mechanism through which aloe-emodin exerts its cytotoxic activity. In detail, the induction of apoptosis by aloe-emodin was related to the activation of caspases cleaves, and then activating downstream caspases.

Keywords: aloe-emodin, apoptosis, leukemia, neuroectodermal tumor

CONTENTS

PHYTOCHEMICAL PROFILE AND PROPERTIES OF *ALOE*

Aloe plants have long been used in traditional medicine for their curative and therapeutic properties and, although many different active compounds have been identified, therapeutic effects were not well correlated to an individual component. According to Anselm (2004) over 300 species of the genus *Aloe* have been identified and several of them are used; however, *Aloe barbadensis* Miller L. (trivially called *A. vera*) and *Aloe arborescens* L. are the most extensively cultivated in the world (Liao *et al.* 2006). *Aloe* plants are perennial and succulent, with leave that release sticky exudates when broken or injured (Akinyele *et al.* 2007). *Aloe barbadensis* has a short stem and lanceolated green leaves with little white blots. *Aloe arborescens* is a plant with higher stem, dark green and curved leaves (Carpano *et al.* 2009).

Various pharmacological activities and therapeutic effects are ascribed to *Aloe*, such as antiinflammatory, antimicrobial, antidiabetic, antioxidant, as well as hypoglycaemic, gastroprotective, immunomodulatory and wound healing effects (Jones 2008; Manoj *et al* 2009).

Many authors believe that the various biological activities related to *Aloe* should be ascribed to a synergistic action of several compounds rather than a single chemical substance (Dagne *et al.* 2000; Hamman 2008). On the other hand, authors agree that two main components can be identified in *Aloe* leaves: a C-glycoside derivative of a 1,8-dihydroxyanthraquinone known as aloin, and a β-polysaccharide fraction containing either a single chain backbone of β(1-4) mannans, and β(1-4) glucomanans with α(1-6) branching, both partially acetylated and having variable molecular weights ranging over three orders of magnitude. Acemannans are probably the most known among polysaccharides belonging to this class of compounds (Reynolds 1985; Boudreau *et al.* 2006; Hamman 2008; Jones 2008).

The *Aloe* leaf can be divided into two main fractions, namely the outer green rind and the inner colorless parenchyma. The rind is rich in 1,8-dihydroxyanthraquinone derivatives and their glycosides, while the parenchyma tissue or pulp seems to be richer in complex carbohydrates (Hamman 2008). Several commercial products are derived from *Aloe*, using the dermal exudates, the gel or the total extracts of the leaves, hence with different chemical composition and biological properties.

It is well known, however, that physical-chemical properties and activity of natural products are a function of the type of extract, species of plant, the raw materials utilised and the conditions of storage (Fanali *et al.* 2010; Pellizzoni *et al.* 2011). Furthermore, plant age and growth conditions (and particularly light), are expected to affect significantly the biosynthesis of secondary metabolites. It is reported that *Aloe* metabolites concentration and cells ultrastructure are different among plants grown under different light irradiance, even this relationship is not clear for aloin (Peaz *et al.* 2000; Li *et al.* 2006).

Among *Aloe* bioactive components, anthraquinones (**Fig. 1**) are *Aloe* secondary phenolic metabolites including C-glucosyl derivatives such as barbaloin (10-glucopyrano-

Received: 16 February, 2012. Accepted: 25 July, 2012.

Invited Mini-Review

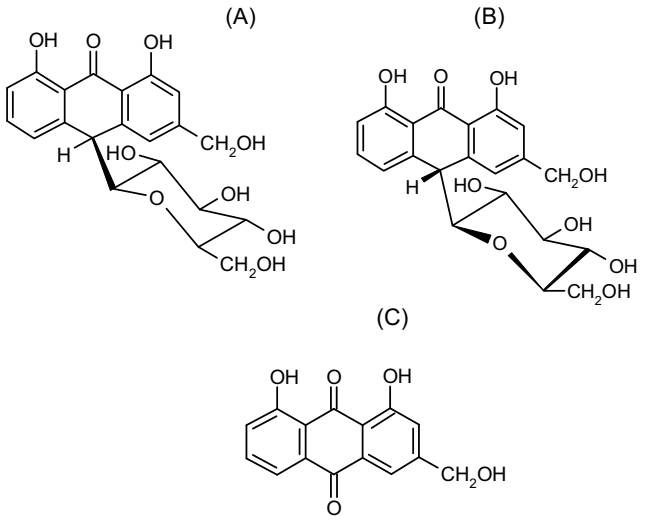

Fig. 1 Chemical structure of the main anthraquinone derivatives of *Aloe*. (**A**) Aloin A; (**B**) aloin B; (**C**) aloe-emodin.

syl-1,8-dihydroxy-3-hydroxymethyl-9-10H-anthracenone), a mixture of the two diastereoisomers aloin A and B, as well as glucose-free compounds such as aloeresin, aloenin and aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione) (Fanali *et al*. 2010). They have been found in the yellowish bitter exudate seeping out from freshly cut leaves and were reported to have cathartic effects, anti-inflammatory effects *in vivo*, to increase the peristaltic movements of the intestinal musculature and also prevent the colon from reabsorbing water (McAnalley 1993; Gutterman *et al.* 2006). It has been proposed that anthraquinones and dihydroxyanthraquinones have direct antimicrobial activity (Wu *et al.* 2006). Other studies have reported on the effect of the anthraquinone aloe-emodin on arylamine *N*-acetyl transferase activity in *Helicobacter pylori* and hence its antimicrobial activity (Wang *et al.* 1998). Anthraquinones showed also antimicrobial activity against *Staphylococcus aureus* strains, *Escherichia coli*, and other microbes probably through inhibition of solute transport in membranes (Hamman 2008; Lone *et al.* 2009; Pellizzoni *et al.* 2012).

ALOE AS ANTICANCER

With the aim of developing novel anticancer drugs characterized by selective targeting and low toxicity, a number of natural compounds that have traditionally been used to treat a variety of diseases for hundreds of years can be taken into account. Among them, *Aloe* anthraquinones have been quite extensively studied with this aim, to treat several tumours both as pure substances and as leaf powder. In fact the anthraquinones aloin A and B, as well as aloe-emodin, are structurally similar to DNA binding drugs such the anthracyclines. Furthermore, both anthraquinones and anthracyclines comprise both glycoside derivatives and aglycones, albeit an anthracenone structure is present in the glycoside derivative aloin, instead of the anthraquinone structure like in all other cases.

Different mechanisms have been postulated regarding the antitumor effect of *Aloe*, including the induction of apoptosis (Lee *et al.* 2001) and a significant elevation of key antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (El-Shemy *et al.* 2010). The relationship between *Aloe* and oxidative status was also investigated by Singh and co-workers (2000). They investigated the effect of *Aloe barbadensis* leaf pulp extract on antioxidant enzymes, glutathione content, lactate dehydrogenase and lipid peroxidation in the liver of mice. The modulatory effect of the pulp extract was also investigated on extrahepatic organs such as lung, kidney and stomach for the activity of glutathione *S*-transferase (GST), DT-diophorase, SOD and catalase (Singh *et al.* 2000). The levels of GST, DT-diaphorase, SOD, catalase, GPx and glutathione reductase were significantly increased in the liver (Singh *et al.* 2000). Treatments caused also a decrease in malondialdehyde, suggesting the role of *Aloe* to protect against pro-oxidant induced membrane damage. The pulp extract was effective in inducing GST, DT-diaphorase, SOD and catalase as measured in extra-hepatic organs. Thus, other organs (lung, kidney and stomach) were favourably influenced in order to detoxify reactive compounds (Singh *et al*. 2000).

Beside *Aloe* anthraquinones and derivatives, other compounds structurally close to them have been investigated for their anticancer properties. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an anthraquinone from the roots and barks of numerous plants, which has been studied as anticancer agent because it was demonstrated to modulate cell cycle in specific oncogene overexpressed cells (Srinivas *et al.* 2007). Its activity against tumours has been related to the modulation of apoptosis, although additional inhibitory effects on angiogenic and metastatic regulatory processes have been postulated as well. Rhein is a major rhubarb anthraquinone, and it is reported to effectively inhibit the uptake of glucose in cancer cells, to cause changes in membrane-associated functions and then to led to cell death (Huang *et al.* 2007; Chen *et al.* 2009). The authors also confirmed the role of emodin and aloe-emodin, two additional anthraquinones which are present also in rhubarb, in controlling tumour proliferation by inducing apoptosis.

Finally, albeit further information is related to the direct anti-cancer efficacy of aloe-derived compounds, it must be noted that a chemopreventive effect of *Aloe arborescens* has been reported as well. The powder of freeze-dried whole leaf has been demonstrated to be able to prevent N-nitrosobis (2-oxopropyl)amine induced pancreatic carcinogenesis in hamster (Furukawa *et al.* 2002). Whole leaf of *Aloe arborescens* was also reported to inhibit the initiation stage of colon carcinogenesis induced by azoxyethane in rat colorectum (Shimpo *et al.* 2001).

Aloe-emodin and neuroectodermal tumours

Pecere *et al*. (2000) extensively reported on the selective *in vitro* and *in vivo* killing of neuroectodermal tumour cells by aloe-emodin, the anticancer activity of which has been related on apoptotic cell death, promoted by a tumour cell-specific drug uptake process. The cytotoxicity of aloe-emodin has been reported by the authors: aloe-emodin selectively inhibited human neuroectodermal tumour cell growth both in tissue cultures and in animal models. Neuroblastoma, pPNET, and Ewing's sarcoma cells were found highly susceptible to this compound, whereas human malignant cells from epithelial and blood-derived cancers, as well as human hemopoietic progenitors and normal fibroblasts, were not sensitive to it. Indeed, the growth of the neuroectodermal cancer cell lines was specifically inhibited, and effective doses ranged between 1 and 13 μM (neuroblastoma and Ewing's sarcoma, respectively). Conversely, epithelial tumours such as cervix carcinoma and colon carcinoma cells, and also T-cell leukemia cells and normal fibroblasts, were almost refractory to the treatment with aloe-emodin. The effective doses for these cell lines ranged from 40 μM for cervix carcinoma cells to 100 μM for T-cell leukaemia cells.

To explain the specific cytotoxic activity of aloe-emodin against neuroectodermal tumour cell lines, the authors investigated its cellular uptake by different cell lines, evaluating the green fluorescence of the compound. The neuroblastoma cells gave rise to an intense fluorescence emission, while no fluorescence was observed for the hemopoietic progenitor cells. With colorectal carcinoma and T-cell leukaemia cell lines, lack of drug uptake was also observed at 37°C.

Microscopic observations after 24 h of incubation indicated that aloe-emodin was present in the cytoplasm of

neuroblastoma cell lines inside endosomes. Nuclear localization of the anthraquinone was readily observed in the sensitive cells at 1 h after treatment. The paper reports also the effects of aloe-emodin on cell cycle and apoptosis of neuroblastoma cells, through flow cytometry over a period of 48 h. Apoptotic cells with fragmented DNA have been observed after 48 h, and typical morphological features of apoptotic cell death (cell shrinkage, membrane blebbing, and nuclear fragmentation) were also exhibited by most cells at TEM analysis.

The anticancer properties were then assessed *in vivo*, in a murine model system. Mice were injected with human neuroblastoma cells and immediately treated with aloe-emodin at a dose of 50 mg/kg/day (the highest concentration compatible with an aqueous solution). The tumour was sensitive to the drug, as shown by a significant reduction ($P < 0.05$) of its growth. Furthermore, when the treatment was delayed until a palpable tumour mass had developed (day 15), tumour growth was halted throughout the period of administration ($P < 0.05$). The human colon carcinoma cell line injected into mice was instead refractory to the treatment, in agreement with the results *in vitro*. No appreciable signs of acute or chronic toxicity were observed in any of the treated animals and no other manifestation of acute toxicity was evident. No structural abnormalities were observed on macroscopic examination in either the treated or control group.

Aloe and leukemia

Grimaudo and co-workers (1997) investigated the effect of purified anthraquinones on sensitive and multidrug resistant leukemia cells. In their preliminary studies (Speranza *et al.* 1994), they reported an antitumor activity *in vitro* of five *Aloe*-derived anthraquinones, namely aloe-emodin, aloin A, aloin B, aloesin and aloeresin extracted from *Aloe barbadensis* Mill. Some authors (Kupchan *et al.* 1976; Lu *et al.* 1989) also reported the possible anticancer effect of this anthraquinone against leukemia cells. On this basis, they evaluated the efficacy of these compounds against leukaemia (both using a sensitive and a multidrug resistant line), using trypan blue exclusion and microscope for their assessments. Aloin A and B, aloeresin and aloesin did not show any cytotoxic activity even at high concentrations. Aloe-emodin, however, did show a reproducible cytotoxic activity but at concentrations much higher than the ones of common anticancer agents such as daunorubicin and etoposide, both tested as a comparison in the same cell line. However, chemotherapy agents were from 11 to 250 times less effective against the resistant cell line, while aloe-emodin was three times more effective in this line. Aloe-emodin exhibited an anti-proliferative effect. The analysis of cell survival curves and of the cell cycle distribution revealed that the cytotoxic activity of aloe-emodin was mainly ascribed to cytostasis.

Other authors studied the anticancer effect of *Aloe* anthraquinones against leukemia (El-Shemy *et al.* 2010). Aloin, aloe-emodin and aloesin purified from *Aloe barbadensis* have been tested against acute myeloid leukemia (AML) and acute lymphocites leukemia (ALL) cancerous cells by the trypan blue cell viability assay. The *Aloe* secondary metabolites showed a significant dose-dependent cytotoxicity against both AML and ALL cells. The authors also observed that treatment of human AML cells with the above mentioned compounds resulted in different internucleosomal DNA fragmentation, hallmark of cells undergoing apoptosis. Aloe-emodin was the most effective compound, followed by aloesin and aloin.

Aloe and Merkel cell carcinoma

Merkel cell carcinoma (MCC) is an aggressive tumor of the skin that mainly affects elderly in sun-exposed regions. MCC cells do express neuroendocrine and epithelial properties, and therefore this tumor is also known as primary endocrine carcinoma. Albeit MCC cells are chemo- and radiosensitive, this tumor has an unfavorable prognosis because conventional therapies provide only temporary benefits.

Fenig *et al.* (2004) evaluated anthraquinones as an alternative therapeutic approach against MCC based on the preliminary observation that these compounds possess selective toxicity against neuroectodermal tumors such as neuroblastoma (Pecere *et al.* 2000). Since that, they firstly observed a significant *in vitro* inhibition of MCC proliferation using a suspension culture (Wasserman *et al.* 2002). A striking inhibitory effect of aloe-emodin on the viable cell number after 72 h of treatment was demonstrated, and the results were statistically significant ($P < 0.02$) starting from 10 μM aloe-emodin. The glycosidic derivative aloin was tested at the same concentrations however no effect was detected.

Their subsequent work targeted to investigate the effect of some anthraquinones, namely emodin, aloe-emodin and aloin on the proliferation of adherent MCC cells. The cells number, evaluated through the sulforhodamine B method (SRB, by which stained cells were read with a microtiter ELISA), evidenced a strong proliferation-inhibiting activity by emodin and aloe-emodin, in a dose-dependent manner. In detail, aloe-emodin was found to be slightly more effective than emodin, while aloin had no effect on cell proliferation.

Aloe and lung squamous carcinoma

The effect of aloe-emodin on human lung squamous carcinoma cell line CH27 has been extensively investigated and reported by Lee and co-operators (2001) in order to clarify the role of this compound as anticancer factor. As far as concerns morphological alterations of the cell line following the treatment, it was evaluated by microscopic inspection: the anthraquinone at a concentration of 40 μM for 18 h induced many apoptotic bodies, while treatment for 36 h resulted in cell death and left cellular wreckage. Following 72 h aloe-emodin exposure, cell death was more extensive. In the same set of experiments, it was demonstrated that the effect was not only time-dependent, but also dose-dependent, while cell decrease in control cultures was negligible.

Treatment with 40 mM aloe-emodin for 24 h resulted in intenucleosomal DNA fragmentation, evidenced by the formation of a DNA ladder, a prove of cells undergoing apoptosis. On this basis, further experiments in which cells were treated with aloe-emodin and then washed free of the anthraquinone, evidenced that tumor cells did not recover and hence the cell death, once triggered, was induced irreversibly.

Aloe combined to chemotherapeutic drugs

Given their *in vitro* and *in vivo* results, Fenig *et al.* (2004) further investigated the combined effect of increasing concentrations of aloe-emodin alone and in combination with some chemotherapy agents such as *cis*-platinum. The effect of aloe-emodin was confirmed and inhibitory effect was statistically significant at all doses tested, with a dose-dependent behavior. Interestingly, the combined treatment of aloe-emodin and *cis*-platinum had an additive growth-inhibitory effect which was prominent mainly with low concentrations of the chemotherapy agent. Similar results were achieved using different agents such as doxorubicin and 5-fluorouracil.

Other authors reported, similarly, that aloe-emodin sensitizes tumor cells to chemotherapeutic drugs (Wasserman *et al.* 2002), against cell lung cancer. These results are promising because, given the combined inhibitory effect prominent at low drug concentrations, the use of aloe-emodin could allow the reduction of effective therapeutic drugs.

It is also reported that emodin, an anthraquinone abundant in plants other than *Aloe* but structurally similar to

aloe-emodin, administered together with gefitinib, can enhance the efficacy of the chemotherapeutic drug and therefore may serve as the basis for a novel and better therapeutic modality in the management of human lung cancer (Chen *et al.* 2009).

It must be noted, however, that some authors (Mijatović *et al*. 2005) found that aloe-emodin reduced the cytotoxic activity of the platinum(II)-based anticancer agent toward a murine fibrosarcoma and a glioma cell line. In detail, the anthraquinone interfered with cisplatinum-triggered activation of extracellular signal-regulated kinase in tumor cells.

ALOE AND APOPTOSIS

Apoptosis is a major form of cell death and it is essential for the maintenance of homeostasis. Apoptosis is characterized by a series of molecular modifications, such as expression and translocation of Bcl-2 family proteins, release of cytochrome *c* from mitochondria, and activation of a family of cysteine protease named caspases. There are at least two major mechanisms by which the caspase cascade - resulting in the activation of effector caspases (caspase-3, -6, and -7) - may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 (Zou *et al.* 1997; Srinivasula *et al.* 1998). Therefore, two typical apoptosis pathways, a receptor-mediated (involving caspase-8) and a chemical-induced (involving caspase-9) apoptosis, have been suggested (Srinivasula *et al.* 1998).

Cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including tumor necrosis factor and chemotherapeutic agents (Liu *et al.* 1996; Kluck *et al.* 1997; Reed 1997).

The Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, Bak, and Bax, are extensively studied and well-characterized regulators of apoptosis: several studies have reported that the release of mitochondrial cytochrome *c* together with the activation of caspase-3 are blocked by anti-apoptosis members of the Bcl-2 family, such as Bcl-2 and Bcl-X , and promoted by pro-apoptotic members, such as Bak and Bax (Kluck *et al.* 1997; Yang *et al.* 1997; Jurgensmeier *et al.* 1998).

The work reported by Lee *et al.* (2001) regarding the effect of aloe-emodin on human lung squamous carcinoma cell line CH27, included a detailed investigation regarding the mechanism by which aloe-emodin induced apoptosis. The authors evaluated the expression of Bcl-2 family proteins through western-blotting. In treated cells an extensive translocation of Bak and Bax proteins from the cytosolic to the particulate fraction was actually observed. The results were consistent with previous observations, in which the pro-apoptotic activity of Bak and Bax was related to their translocation from cytosol to mitochondria (Shimizu *et al.* 1999; Eilon *et al.* 2000). In fact, Bak and Bax are reported to target mitochondria outer membrane channel and allow cytochrome *c* to move toward cytosol. On this basis, the authors also verified that cytochrome *c* increased its abundance after treatment with aloe-emodin.

Western blotting evidenced also a decreased expression of Bag-1 protein, which has been suggested to be an anti-apoptotic protein by several authors (Yang *et al.* 1999a, 1999b; Hayashi *et al.* 2000).

Regarding the effect of treatment on caspases, it was reported that preform of caspase-3 and caspase-9 decreased significantly (Lee *et al.* 2001). The activation of caspases cleaves and activates downstream caspases then inducing apoptosis. In particular, Lee *et al.* (2001) reported that caspase-9 may be the upstream activator of caspase-3 during aloe-emodin induced apoptosis in the CH27 cell line. Treatments also resulted in proteolysis of caspase substrates, hence confirming further the role of these proteases in the induced apoptosis.

Other authors confirmed that anthraquinones can induce apoptosis in tumoral cells. Rhein, an anthraquinone derived from rhubarb, induced dose- and time-dependent increase in caspase-9-mediated apoptosis of human breast cancer cells (Chang *et al.* 2012). Emodin, another anthraquinone from rhubarb, has been reported to induce apoptosis in tumor cells as well (Srinivas *et al.* 2007).

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