Original Article

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Evaluation of natural anthracene-derived compounds as antimitotic agents

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ABSTRACT: Plants that contain anthracene-derived compounds such as anthraquinones have been reported to act as anticancer besides their use for millennia to treat constipation, but the mechanism of action is still unfolding. Therefore we pursue this study to explore a new horizon in the anticancer property of these agents with relevance to mitotic arrest. To achieve this goal, the antimitotic activity of a series of naturally occurring anthracene-derived anthraquinones including anthrone, alizarin (1,2-dihydroxyanthraquinone), quinizarin (1,4-dihydroxyanthraquinone), rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), emodin (1,6,8-trihydroxy-3-methyl-anthraquinone), and aloe emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone) were evaluated using *Allium cepa* root tips. Initial results revealed that the mitosis was inhibited after 3, 6, and 24 h, respectively, of incubation with 500, 250, and 125 ppm of each compound in a dose-dependent manner. Furthermore, alizarin at 500 ppm was proved to be the most active compound to arrest the mitosis after 24 h followed by emodin, aloe emodin, rhein, and finally quinizarin. Interestingly, this inhibition of mitosis was irreversible in root tips incubated with each compound at concentration of 500 ppm but not with 250 ppm or 125 ppm, where the roots regained their normal mitotic activity after 96 h post-incubation in water. This re-evaluation of an old remedy suggests that several bioactive anthraquinones possess promising anti-mitotic activity that may have the potential to be lead compounds for the development of a new class of multifaceted natural anticancer/antimitotic agents.

Keywords: Antimitotic, *Allium cepa*, anthraquinones

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1. Introduction

Every year almost 9 million cancer cases are newly diagnosed in developing countries where cancer incidence continues to increase at alarming rates. According to cancer society statistics, at least one third of these individuals are not expected to survive the disease, making cancer the most prevalent cause of death (*1*). Systemic chemotherapy forms the mainstay of cancer treatment, and agents that disrupt mitotic spindle assembly, so called 'anti-mitotics', have emerged as a new and very promising strategy in treating a wide variety of cancers. Traditional anti-mitotic agents include microtubule toxins such as taxol, other taxanes, and the vinca alkaloids, all of which have proven successful in the clinic. However, these compounds act not only on proliferating tumor cells, but exhibit significant side effects on non-proliferating cells including neurons that are highly dependent on intracellular transport processes mediated by microtubules. On top of that, patient response remains highly unpredictable, drug resistance is common, and in addition, toxicity is a problem (*2,3*). To address these problems, the search has intensified for more successful therapies like the classical anti-microtubule drugs, while avoiding some of the adverse side effects. Because there is still no safe anti-mitotic agent, the use of nutritional therapy in the area of drug discovery proved to be an efficient, safe, and economic tool in health care.

During the last decade, it was reported that many herbal formulas-containing anthraquinones were successfully used for treatment of cancer. Emodin, aloe emodin, and rhein, the most extensively studied anthraquinones, have been reported to inhibit proliferation in breast, lung, cervical, colorectal, and prostate cancers cells (*4-7*) with little or no cytotoxic effects in several normal cells, such as human fibroblast-like lung WI-38 cells, rat heart endothelial cells, rat hepatic stellate cells, and rat hepatocytes (*8*). However, the underlying molecular mechanism(s) involved in the anticancer effects of anthraquinones is still unfolding. As such, in the present study, an expansion has been made to explore the new horizon in anticancer properties of certain naturally available anthraquinones (Figure 1) with

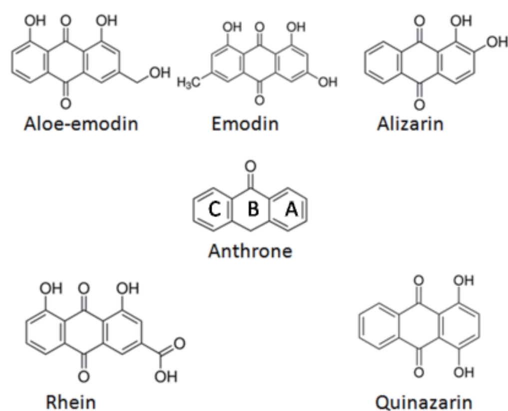

Figure 1. Structural characteristic of natural anthracene-derived compounds, anthrone, aloe emodin, emodin, alizarin, quinizarin, and rhein.

relevance to mitotic arrest. The approach chosen here relies on *in vitro* design since *in vivo* testing to evaluate mitosis is often expensive, time consuming, and requires approval by ethical committees, particularly with mammalian models. Preliminary antimitotic screening in the current study was conducted using the *Allium cepa* root tip assay, a reliable *in vitro* system, which reflects specific effects and allows the selection of promising antimitotic prototypes. This test was initially used by (*9*) in studies on the effect of plant extracts and various chemical compositions on meristematic cells. Up to now, it has been widely used for detection of cytostatic, cytotoxic, and mutagenic properties of different compounds, including anticancer drugs of plant origin (*10,11*). The development and acceptance of such simple bioassays, convenient to use in-house, with a paucity of research funds, are urgent in third world nations, where cultural folk medicines are strongly used safely and effectively to treat human cancers.

2. Materials and Methods

2.1. *Materials*

Anthraquinone, anthrone, alizarin (1,2-dihydroxy-anthraquinone), quinizarin (1,4-dihydroxy-anthraquinone), rhein (4,5-dihydroxy-anthraquinone-2-carboxylic acid), emodin (1,6,8-trihydroxy-3-methyl-anthraquinone), and aloe emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone) were kindly provided by Prof. Dr. A. El-Gaml, Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

2.2. *Methods*

In this study, a modification of Podbielkowska's assay (*12*) was introduced as a simple method to evaluate the effect of drugs on the mitotic process. In order to eliminate any significant differences during sampling, a zero time sampling for all bulbs was examined under the same conditions. Therefore no individual variation was assumed for each experiment. *Allium cepa* L. root tips were selected as a model because their chromosomes are small in number (16 pairs), large in size, and easily examined using the light microscope. The roots were fixed and stained with acetocarmine 1% according to the method mentioned above. Acetocarmine stained both DNA and RNA so nearly all parts of the cell were colored but the chromosomes were stained most heavily because some of the RNA had been leached out by HCL. Mitotic percent was calculated in treated roots and statistically compared with that of controls.

2.2.1. *Growing A. cepa L. bulbs*

A. cepa L. bulbs were grown in 250 mL dark jars filled with tap water at room temperature. The water in the jars were continuously aerated and changed daily until the roots reached 2-3 cm.

2.2.2. *Incubation of compounds*

Three bulbs were transferred to the control solution (tap water only) and four others were transferred to each test solution for 24 h. Compounds in this study were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and the volume was made up to 1 L with tap water to produce three concentrations; 500, 250, and 125 ppm. Control solution was prepared by mixing 1 mL of DMSO with tap water to produce 1 L of this solution. All prepared solutions were protected from light during this study. Five roots of three analogous onions were collected after 3, 6, 12, and 24 h incubation. Postincubation of *A. cepa* L. bulbs in tap water was continued for the next 24 and 96 h in the dark to monitor for possible cell recovery.

2.2.3. *Microscopical examination*

The roots were collected, fixed in 95% ethanol:acetic acid (3:1, v/v) for 6-12 h and washed with 70% alcohol. Then they were immersed in ethyl alcohol 95%:1 N hydrochloric acid (1:1, v/v), fixed again for 5 min, squashed and finally stained with 1% acetocarmine.

2.2.4. *Calculations*

For each root sample, the number of meristematic and total cells present in 5 fields was counted using high power (×100) light microscopy. Results for 30 readings were tabulated at different concentrations and times of incubation. The partial mitotic index is given as the number of cells in the course of division as a percentage of all meristematic cells (*13*). The percent mitosis (the number of mitotic cells per one hundred total cells) was calculated for both control and examined samples and statistically compared.

2.3. *Statistical analysis*

The results were expressed as mean ± SE. All analyses were performed with the GraphPad Prism 3 package. Intergroup comparison was made by an ANOVA test. Thereafter, data were checked for skewedness, and an unpaired *t*-test was performed if the distribution of the values was Gaussian. If the distribution was not normal, a Mann-Whitney test was used. *p*-values less than 0.05 were considered to be statistically significant.

3. Results

The results revealed that all tested anthraquinones markedly caused a retardation and/or an inhibition of mitotic activity observed in the root tips of *A.cepa*. This decrease in the mitotic activity was directly proportional to the increase of the compound concentration or prolongation of incubation time. Generally, the inhibition of mitosis was significantly ($p < 0.05$) observed after 3 h incubation with 500 ppm, 6 h incubation with 250 ppm, and after 24 h incubation with 125 ppm. Furthermore, cell recovery did not occur with 500 ppm but occured with 250 ppm and 125 ppm after 96 h post-incubation in water.

Individually, Figure 2 shows the mean mitotic index (counted as percentage of control, 0 ppm) in root tip meristems of *A. cepa* during 24 h incubation in the different concentrations of anthrone, which generally exists in the fresh plant and then is oxidized by an anthrone oxidase to anthraquinon. This 24 h-incubation was followed by 96 h post-incubation in water. As shown in Figure 2, anthrone at 500 ppm caused ~43% inhibition of mitotic activity after 24 h incubation, while 125 ppm of anthrone solution showed the least retardation effect, 22% inhibition of mitotic activity. In addition, it was observed that 500 ppm anthrone solution prevented formation of new healthy roots while this effect decreased gradually in 250 ppm and 125 ppm.

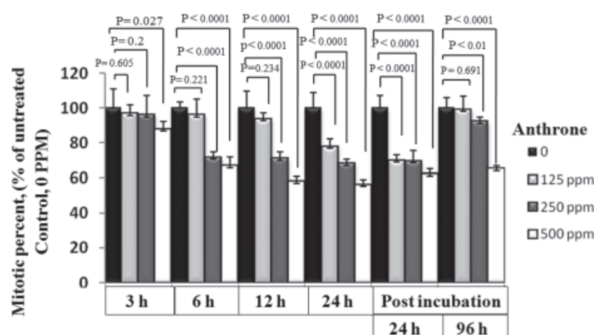

Figure 2. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of anthrone, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

In light of anthron's antimitotic effect, we next sought to determine whether the addition of functional groups would modify this antimitotic behavior of anthraone. Moving over the molecular structure of the selected anthraquinones, we found that introduction of a hydroxyl group in ring A of oxidized anthrone significantly enhanced the anti-mitotic effect of anthraquinones as depicted in Figures 3-5 for aloemodin, emodin, and

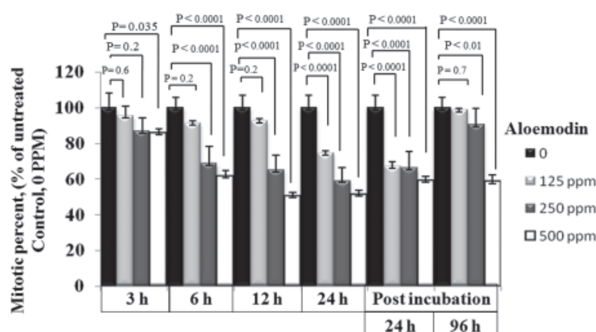

Figure 3. Mean mitotic index of *A. cepa* meristem cells in the control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of aloe emodin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

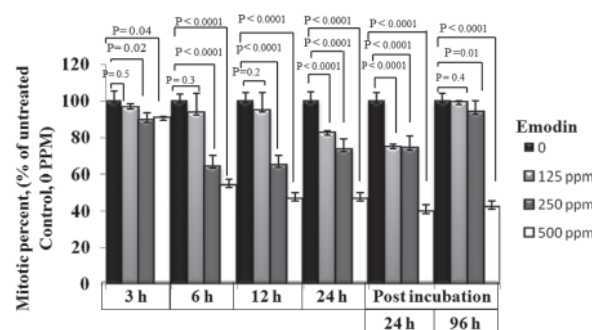

Figure 4. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of emodin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

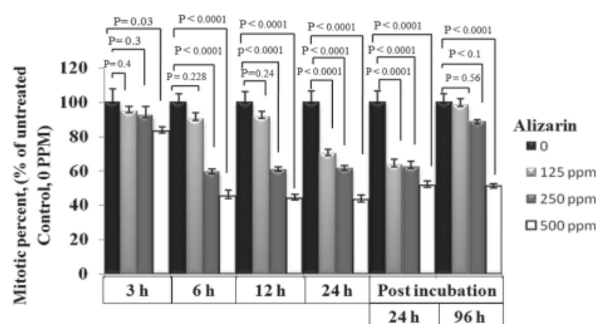

Figure 5. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of alizarin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

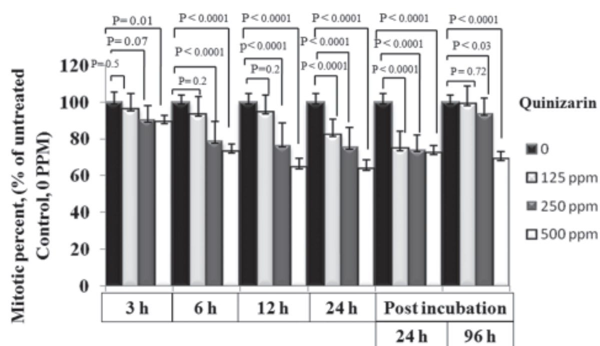

Figure 6 Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of quinizarin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

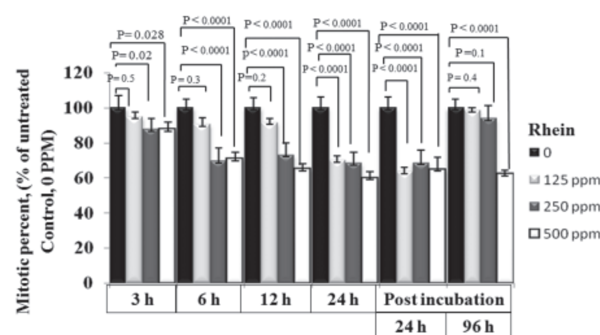

Figure 7. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of rhein, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

alizarin, respectively. In Figure 3, aloe emodin, which has one OH group in ring A, caused ~49% inhibition of mitotic activity of root tip meristems after 24 h incubation at a concentration of 500 ppm. Proceeding further, the addition of a second OH group in ring A resulted in a substantial increase in anti-mitotic activity as illustrated by emodin, which showed ~53% inhibition of mitotic activity of root tip meristems after 24 h incubation at a concentration of 500 ppm (Figure 4). Furthermore, the addition of a second OH in an ortho-substitution had a greater influence on the anti-mitotic effect than substitution in the *meta*-position. This is clearly manifested in alizarin in which the antimitotic activity was raised to 56% after 24 h at a concentration of 500 ppm (Figure 5). However, the addition of a second OH in a *para*-substitution (quinizarin) caused a dramatic decrease in the antimitotic activity to 35% (Figure 6). Likewise, introducing a carboxylic group into ring A also reduced the anti-mitotic effect to 40% as manifested in rhein (Figure 7). One-way ANOVA was used to test for statistical differences in the anti-mitotic effect of 500 ppm after 24 h, and the means were significantly different across the samples ($p < 0.05$) (Figure 8).

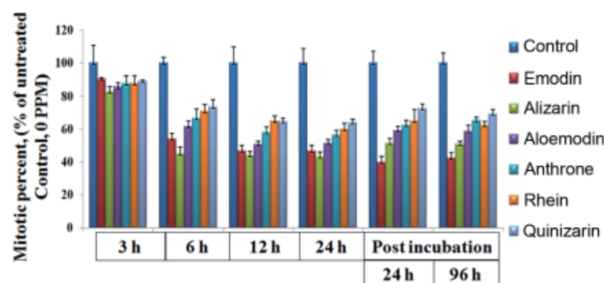

Figure 8. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in the different concentration (125, 250, and 500 ppm) of anthrone, aloe emodin, emodin, alizarin, quinizarin, and rhein, respectively, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

4. Conclusion

For many years, laboratories from all over the world have been working on finding effective therapies for cancer, a disease of our civilization. Drugs that interfere with the normal progression of mitosis belong to the most successful chemotherapeutic compounds currently used for anti-cancer treatment. In spite of their clinical success, some limitations remain, such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance conferred by multidrug resistance transporters and tubulin mutations (*14-16*). Therefore, there has been great interest in identifying novel antimitotic agents with a new mode of action and improved pharmacology profiles especially concerning reduced toxicity. The reemphasis of natural products in drug discovery has been the subject of recent research in cancer drug discovery and development, and has proved to be a valuable, effective, and inexpensive approach (*17*). In the current study, we put forward a new concept pertaining to the use of natural anthracene-derived anthraquinones as potential anti-mitotic agents.

Anthraquinones are quinone derivatives of anthracene found in many species of medicinal plants that possess a plethora of biological and pharmacological properties. For years, much interest has been placed in the development and use of anthraquinones and its derivatives for the prevention and treatment of cancer. Emodin and aloe emodin, the most abundant anthraquinones in rhubarb, are capable of inhibiting cellular proliferation, induction of apoptosis, and prevention of metastasis. However, both components were found to be able to potentiate the anti-proliferation of various chemotherapeutic agents (*18*). To extend knowledge of the antineoplastic role of anthraquinones, the current study opens a new horizon of understanding their properties in fighting cancer by shedding light on their ability to interfere with mitosis. The anti-mitotic activity was evaluated using the *A. cepa* root meristem model, commonly known as the allium assay. This assay

is a rapid, highly sensitive, and reproducible bioassay for detecting antimitotic events (*19*).

Among anthraquinone derivatives tested here hydroxylanthraquinones are also a common structural moiety of adriamycin and mitoxantrone (common antitumor drugs). It is known that hydroxy- anthraquinones containing a poly-aromatic ring structure can bind to DNA by intercalation and stacking between the base pairs of DNA double helices (*20*). Given these properties, hydroxyanthraquinones represent promising anticancer agents. This input has originated partly from a study showing that alizarin, 1,2-dihydroxy-9,10-anthraquinone, inhibited the growth of syngeneic tumors in treated recipient animals (*21*). These initial observations have been supported by the *in vitro* and *in vivo* antineuroectodermal tumor activity of aloe emodin, a hydroxyanthraquinone present in *Aloe vera* leaves, while no appreciable toxic effects were observed on the animals (*22*). Moreover, these findings were reinforced by recent studies showing that emodin, 6-methyl-1,3,8-trihydroxy-anthraquinone, has a potential anti-tumor effect on pancreatic cancer *via* its dual role in promotion of apoptosis as well as its suppression of angiogenesis, through regulating the expression of NF-κB-regulated angiogenesis-associated factors (*23*). All of this seemingly overwhelming evidence has shaped the concept of hydroxyanthraquinones as antineoplastic agents and several mechanisms have been proposed regarding their anticancer activity. One refers to the ability of the drug to intercalate DNA (*20*) and inhibit DNA topoisomerase II (*24*). Another refers to the ability of the drug to produce free radicals and consequently to cleave DNA (*25*). Nevertheless, this study is the first to demonstrate the antimitotic activity of this group of natural products, suggesting an additional mode of action for hydroxyanthraquinones.

It is noteworthy to mention that although the basic chemical structures of various anthraquinones are similar, the specific functional groups attached at specific positions, particularly for the hydroxyl group, can confer remarkably different bioactivities to the resulting compounds. This notion is exemplified here by reporting that alizarin, which is the only hydroxyquinone having a hydroxyl group in a β position, to be the most active compound to arrest mitosis followed by emodin, aloe emodin, rhein, and finally quinzarin. To the contrary, 1-hydroxyanthraquinone has been reported to be carcinogenic (*26*). Recently it has been reported that lucidin, a hydroxyanthraquinone derivative present in this plant, is mutagenic in bacteria and mammalian cells. In addition, formation of DNA adducts in tissue culture and mice after treatment with this compound has been documented (*27*).

In the current study, analysis of changes in the mitotic index when treated with the selected series of anthraquinones showed that depending on their concentration, the mitotic activity was reduced or inhibited. These observations are supported in existing results concerning animal cells as well as in several human cancer cells (*e.g.*, hepatocellular, lung, breast, esophagus, and gastric) *in vitro* (*4-7*). They induce apoptosis, have an anti-angiogenesis effect, and inhibit the invasion and metastasis of tumor cells (*23,28-30*). Moreover, the antitumor activity of anthraquinone was compared to that of daunorubicin, which is structurally different from anthraquinone but also contains a quinone moiety (*24*).

In conclusion, with the continuing need for novel drug-like lead compounds against the increasing number of ever-more-challenging molecular cancer targets, the chemical diversity derived from natural products will be increasingly relevant for the future of drug discovery. Therefore, the activity of the studied naturally anthracene-derived series in inhibiting mitosis followed by lack of recovery in post-incubation make them potential leads for antimitotic agents.

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