

Chemopreventive Activity of Naphthoquinones from *Alkanna tinctoria* (L.) Tausch in Human Colorectal Cancer Cells

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(3), and 5-methoxyangenyalkannin (4) showed strong anti-proliferative effects, while compound 4 showed the most potent effects. Compound 4 arrested cancer cells in the S and G2/M phases, and significantly induced cell apoptosis. The apoptotic effects of compound 4 were supported by caspase assay and docking analysis.

Key words: *Alkanna tinctoria*; Naphthoquinones; Alkannin derivatives; Novel compounds; Antiproliferation; Cell cycle; Apoptosis; caspase 9; Docking analysis; Colorectal cancer

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ABSTRACT

Botanically derived natural products have recently become an attractive source of new chemotherapeutic agents. To explore active anti-colorectal cancer compounds, we performed phytochemical studies on *Alkanna tinctoria* and isolated eight quinone compounds. Using spectroscopical and physicochemical methods including high-resolution mass spectrometry and NMR spectra, compounds 1, 2, 3, 5 and 6 were identified as alkannin, acetylalkannin, angelylalkannin, dimethylacryl alkannin and arnebifuranone, respectively. Novel compounds 4, 7, and 8 are named as 5-methoxyangenyalkannin, alkanfuranol and alkandiol, respectively, and structurally elucidated based on extensive spectroscopic evidence. The anti-proliferative effects of these eight compounds on HCT-116 and SW-480 human colorectal cancer cells were determined by the MTS method. Cell cycle and apoptosis were determined using flow cytometry. Enzymatic activities of caspases were determined by colorimetric assay, and interactions of compound 4 and caspase 9 were explored by docking analysis. Among the eight compounds, alkannin (1), angelylalkannin

INTRODUCTION

Many countries have been moving towards an aging society, especially Japan changed the construction of population quickly resulting in a rapidly increasing prevalence in lifestyle diseases, including colon cancer. Japanese patient survey by the Ministry of Health, Labour and Welfare reported 235,000 colon cancer patients in 2015, and this number is increasing year by year. It is said that the change of dietary life style from the typical Japanese food to the westernized style food induced its increase. Although the cancer chemotherapy has been widely developing recently, some natural product support, having wide spectra of bioactivity, however mild, are required. This is the reason why natural products having evident activities for cancers are particularly desirable in Japan. Considering such recent health circumstances in Japan, we selected saffron for preventing and increasing quality of life against cancers, and reviewed previously^[1]. We have been searching a second candidate as anti-colorectal cancer active natural product, and reached to *Alkanna tinctoria* (L.) Tausch

(Boraginaceae) which is widely distributed in Europe and Western Asia because a previous review informed a wide pharmacological activity of naphthoquinones like ulcers, inflammation, wounds^[2-4].

It is interesting that a number of enantiomers of alkannin like shikonin were isolated from the same family *Lithospermum erythrorhizon* Siebold et Zuccarini which grows in Japan, Korea and China^[5]. Both *A. tinctoria* and *L. erythrorhizon* have the long historical evidences which were listed in Material Medica and Shennong Ben Cao Jing as important medicines in around one century, individually. This is the reason why we select *A. tinctoria* as a second candidate because its constituents might be relatively safety.

In this review, we performed a systemic phytochemical isolation on *A. tinctoria*. Eight naphthoquinones were isolated and they were structurally elucidated including three novel components. The anti-proliferative effects of the eight compounds on human colorectal cancer cells were determined. The effects of these compounds on the cell cycle and apoptosis were evaluated. To further explore the potential mechanisms of apoptosis, a structure-based docking model was simulated to get an inside view of ligand-receptor interaction.

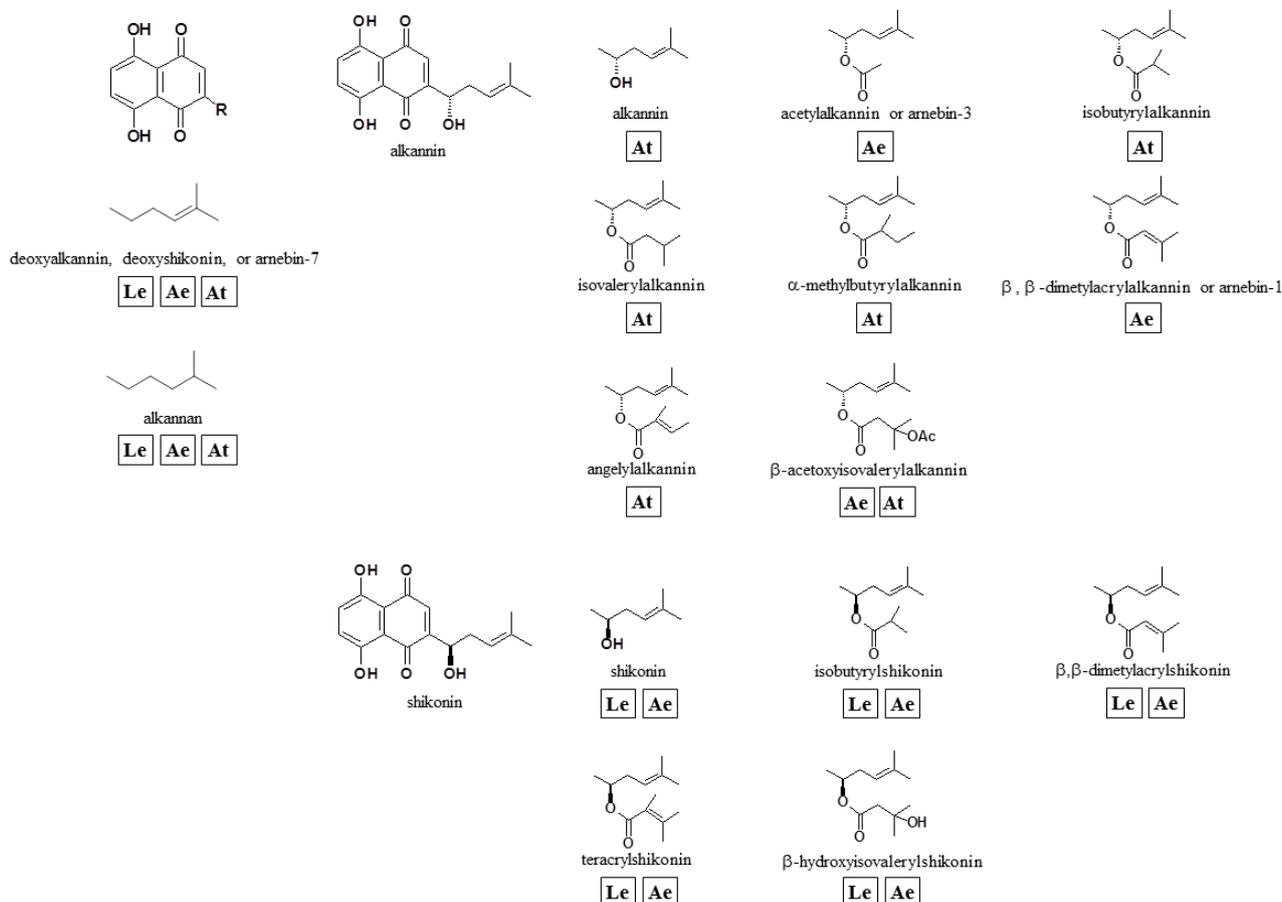
RESOURCES OF NAPHTHOQUINONES

As previously indicated two species of Boraginaceae, *A. tinctoria* and *L. erythrorhizon* contain typical naphthoquinones like alkannin and shikonin, respectively which are the enantiomers each other. Since one other species, *Arnebia euchroma* grown in China is available in Japanese market, we surveyed components in three species

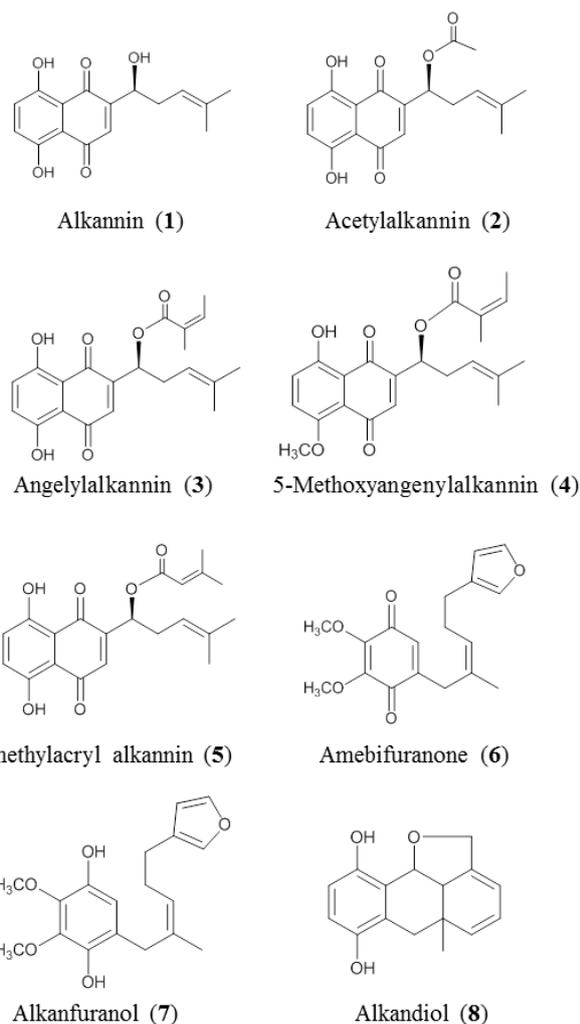
in references^[2,5-11]. Figure 1 indicated the distribution of components contained in three species. It becomes evident that the components having no hydroxyl or ether group on C-1' position in a molecule in the early stage of biosynthetic pathway like deoxyalkannin, deoxyshikonin and arnebin-7 are contained in three species. However, after induction of oxygen function on C-1' position S- and R-enantiomers were separately contained in *A. tinctoria* and *L. erythrorhizon*, respectively. On the other hand *A. euchroma* contains both enantiomers as shown in Figure 1. This evidence suggested that *A. euchroma* might be a hybrid between *A. tinctoria* and *L. erythrorhizon* also supported by morphological characterization. From this result it makes it clear that species can be selected for the isolation of individual enantiomer depends on the purpose of use.

ISOLATION OF NAPHTHOQUINONES FROM *A. TINCTORIA*

Alkannin (1) and naphthoquinone derivatives were concentrated in the pigment fraction of *A. tinctoria*, which is obtained by partition between hexan/CHCl₃ and water^[12]. There have been identified more than 15 alkannin analogs. The structures isolated were characterized by chemical and physicochemical methods, especially NMR and MS spectra. Of them, alkannin (1) and angelylalkannin (3) were major pigment components. We had succeeded to elucidate structures of more 6 naphthoquinones, acetylalkannin (2)^[2], 5-methoxyangelylalkannin (4), dimethylacryl alkannin (5)^[2,13], amebifuranone (6)^[14], alkanfuranol (7) and alkandiol (8) as shown in Figure 2.



Figures 1 Survey of related naphthoquinones in 3 species, *Lithospermum erythrorhizon* (Le), *Arnebia euchroma* (Ae) and *Alkanna tinctoria* (At).

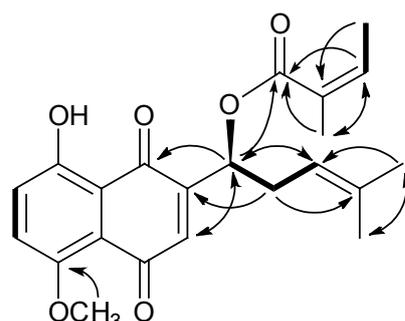


Figures 2 Isolated naphthoquinones from *A. tinctoria*.

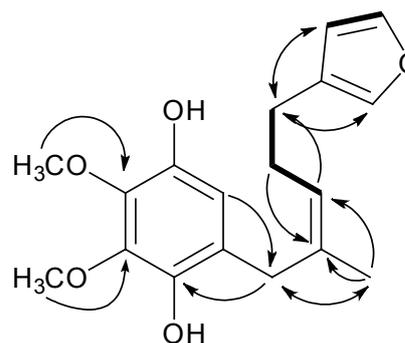
Among them 4, 7 and 8 are novel naphthoquinones. It became evident that the stereochemistry of C-1' of 1, 4-naphthoquinone derivatives from *A. tinctoria* like alkannin is specific as *S*-configuration which is an enantiomer of shikonin which has *R*-configuration as reported previously^[5].

Compound 4, a red solid, gave a quasimolecular ion peak at m/z 385.1653 $[M + H]^+$ in the high-resolution electrospray-ionization time-of-flight mass spectrometry (HRESITOFMS). The ¹H- and ¹³C-NMR spectra of 4 exhibited proton signals of two ortho-coupled and one singlet aromatic protons and two conjugated carbonyl groups, indicating 4 belongs to 1,4-naphthoquinone derivatives like alkannin (1). In addition, signals of an aromatic methoxy (5-OCH₃) were observed. Further analyses of ¹H and ¹³C-NMR data suggested the presence of a 4-methylpent-3-en-1-ol side chain and an angenoyl group, which are similar to angelylkannin (3)^[13]. Furthermore, the overall structure of 4, especially linkage sites, was assigned by ¹H-¹H COSY, HMQC, and HMBC, respectively (Figure 3). Having established the structure of 4, like alkannin (1), the absolute configuration of C-1' was assigned to be *S* using optical rotation value^[13]. Hence, the structure of 4 were identified as 5-methoxyangelylkannin [1-(8-hydroxy-5-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enylangelyloate] (Figure 2).

Alkanfuranol (7) was obtained as a purple solid and had the molecular formula C₁₈H₂₂O₅ on the basis of HR-MS measurement.



Figures 3 H-H COSY (bold lines) and key HMBC correlations (arrows) supporting the structures of 4.



Figures 4 H-H COSY (bold lines) and key HMBC correlations (arrows) supporting the structures of 7.

The ¹H NMR spectrum of 7 showed five aromatic/olefinic signals [δ 5.36 (t, $J = 6.8$ Hz, H-9), 6.28 (br s, H-13), 6.40 (s, H-6), 7.23 (br s, H-15), and 7.34 (br s, H-14)], three methylene groups [δ 2.36 (q, $J = 7.2$ Hz, H-10), 2.48 (t, $J = 7.2$ Hz, H-11), and 3.29 (s, H-7)], one vinyl methyl [δ 1.62 (d, $J = 1.2$ Hz, H-16)], and two methoxy groups [δ 3.89 (s, 3-OCH₃) and 3.91 (s, 4-OCH₃)], respectively. The ¹³C NMR spectrum showed sixteen signals of a typical 3,4-dimethoxy-benzodihydroxyquinone [δ 121.1 (C-1), 140.5 (C-2), 137.4 (C-3), 139.0 (C-4), 141.7 (C-5), and 109.9 (C-6)], a mono-substituted furan ring [δ 124.9 (C-12), 111.1 (C-13), 142.6 (C-14), and 138.9 (C-15)], and isoheptenyl moiety. The ¹H and ¹³C NMR data of 7 were quiet similar with those of amebifuranone (6) apart from the 3,4-dimethoxy-benzodihydroxyquinone moiety^[14]. Furthermore, the structure of 7 was confirmed by the COSY and HMBC spectra (Figure 4). Consequently, the structure of alkanfuranol (7) was unambiguously established as depicted in Figure 2.

Compound 8, a white amorphous powder, had the molecular formula C₁₆H₁₆O₃ on the basis of HRESIMS experiment. The ¹H NMR spectrum of 8 showed signals of two aromatic protons [δ 6.62 (d, $J = 8.8$ Hz, H-5), 6.53 (d, $J = 8.8$ Hz, H-6)] typical of 2,3-disubstituted benzodihydroxyquinone, three conjugated olefinic protons [δ 5.99 (dd, $J = 17.6, 10.4$ Hz, H-11), 5.10 (dt, $J = 10.4, 1.2$ Hz, H-12), and 5.04 (dd, $J = 5.6, 1.2$ Hz, H-10)], one oxymethine proton [δ 4.97 (d, $J = 6.4$ Hz, H-7)], one oxymethylene proton [δ 4.36 (s, H-15)], and one methyl group [δ 0.90 (s, H-16)], respectively. The ¹³C NMR spectrum showed sixteen signals including six aromatic carbons of a benzodihydroxyquinone [δ 148.6 (C-1), 121.9 (C-2), 124.5 (C-3), 150.5 (C-4), 116.2 (C-5), and 113.8 (C-6)], four conjugated olefinic carbons [δ 150.0 (C-9), 107.6 (C-10), 113.0 (C-11), and 148.6 (C-12)], two oxygenated carbons [δ 76.8 (C-7) and 71.9 (C-15)]. The ¹H and ¹³C NMR data of 8 were quiet similar with those of rhizonone, which was isolated from *L. erythrorhizon*, apart from the benzodihydroxyquinone moiety^[15]. Furthermore, the planar structures

of 8 were confirmed by COSY and HMBC experiments. As shown in Figure 5, the 1H-1H COSY experiment on 8 indicated the presence of partial structures written in bold lines; and in the HMBC spectrum, the long-range correlations were observed between the following protons and carbons: H-5 and C-1,3; H-6 and C-2,4; H-7 and C-1,3,9; H-15 and C-8,10; H-16 and C-8,12,14. Hence, the planar structure of alkandiol (8) was established as depicted in Figure 1. The absolute stereochemistry of 8 has not achieved yet although its NOESY spectrum was recorded and the cross-peaks of H-7/H-8 and H-7/H-16 were observed (Figure 5).

The occurrence of alkandiol (8) in *A. tinctoria*, the diol form of rhizonone, which is a novel metabolite putatively derived from geranylhydroquinone, has further suggested the similar biosynthesis pathway between alkannin and shikonin derivatives^[7,15,16].

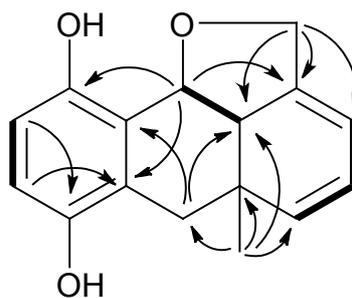
ANTI-PROLIFERATIVE EFFECTS OF 8 COMPOUNDS ON HUMAN COLORECTAL CANCER CELL LINES, HCT-116 AND SW-480 CELLS

Previous reports informed that naphthoquinones had anti-cancer activity^[17,18]. We evaluated the anti-proliferative effects of eight compounds using two human colorectal cancer cell lines, HCT-116 and SW-480. The eight compounds showed different anti-proliferative effects on the two cancer cell lines. At the tested concentrations (3-50 μ M), compound 8 did not inhibit cancer cell growth on either of the two cancer cell lines. Compounds 1 and 4 showed strong anti-proliferative effects. At concentrations of 3 μ M and 10 μ M, compound 1 inhibited cell growth by 29.7% and 59.3%, while compound 4 inhibited cell growth by 43.1% and 77.4%, respectively. Compared to the other seven compounds, compound 4 showed the strongest anti-proliferative effects on HCT-116 cells. Compounds 3 and 5 showed moderate effects; when treated with 30 μ M the cancer cell growth was inhibited by 77.8% and 69.6%, respectively. Regarding the effects on HCT-116 cells, Compounds 6 and 7 showed relatively weak anti-proliferative effects, and at the concentration of 50 μ M, HCT-116 cell growth was inhibited by 91.7% and 92.5%, respectively. Compound 2 showed some anti-proliferative effects on SW-480 cells, but such effects were not observed in HCT-116 cells. Compound 6 showed different potential for cancer cell growth inhibition. The IC₅₀ of compounds 1-8 on HCT-116 cells are: 7.8, >50, 16.3, 4.4, 20.4, 34.6, 39.9, >50 (μ M), respectively.

Isolated compounds showed different anti-proliferative effects on SW-480 cells. However, the sensitivity of certain compounds on SW-480 cells was not similar to HCT-116 cells. Compounds 2 and 6 showed relatively weak anti-proliferative effects; compound 5 showed a moderate effect; compounds 1, 3, and 4 showed relatively strong effects, while compound 4 showed the strongest cancer cell inhibition effect on SW-480 cells. The IC₅₀ of compounds 1-8 on SW-480 cells are 15.1, 34.1, 17.0, 9.6, 22.9, 40.0, 19.0, >50, respectively. Data from both the HCT-116 and SW-480 cell lines suggested that the strongest anti-proliferative effect was observed when using compound 4, a novel isolated compound.

EFFECTS OF 8 COMPOUNDS ON CELL CYCLE DISTRIBUTION OF SW-480 CELLS

To examine whether proliferation in treated cells decreased because of cell cycle arrest at a specific phase, cell cycle profiles were



Figures 5 H-H COSY (bold lines) and key HMBC correlations (arrows) supporting the structures of 8.

determined using flow cytometry after staining with PI. Besides compound 8, the other seven compounds induced cell cycle profile changes in different patterns. After treatment with 10 μ M of compounds 5 and 7, the SW-480 cells were arrested mainly in the G2/M phase. Compounds 1, 2, 3, 4, and 6 decreased the percentage of cells in the G1 phase and increased cell proportions in both the S and G2/M phases. S phase cells became a major group, especially in treatment with higher concentrations. Among the 8 compounds, because compound 4 showed most potent anti-proliferative effect, and also possessed marked activity on S and G2/M phase arrest, the expressions of proteins that control S and G2/M phase transition were evaluated. Compared to the control, expression of cyclin B1 and cdc2 were decreased dose-dependently. On the other hand, compound 4 significantly up-regulated p21, while slightly increasing cdk2. Our results were consistent with the role of these proteins in the regulation of the S and G2/M transition^[19,20]. Data from this study suggests that those seven compounds arrested SW-480 cells in the S and G2/M phases, and compound 4 induced S and G2/M arrest through the modulation of cell cycle-regulating proteins.

APOPTOTIC INDUCTION EFFECTS OF 8 COMPOUNDS ON SW-480 CELLS

Apoptosis is a highly regulated programmed cell death process in which cells undergo inducible nonnecrotic cellular suicide, and thus plays an important role in anticarcinogenesis. Apoptosis is characterized by distinct morphological changes such as cell shrinkage, chromatin condensation, plasma membrane blebbing, and oligonucleosomal DNA fragmentation^[21]. To explore the potential mechanism through which the tested compounds inhibit cell growth, cell apoptosis was assayed by flow cytometry after staining with double annexin V and PI in SW-480 cells. Annexin V can be detected in both the early and late stages of apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both annexin V and PI; early apoptotic cells were positive for annexin V and negative for PI; late apoptotic or necrotic cells displayed both positive annexin V and PI (upper right quadrant); non-viable cells which underwent necrosis were positive for PI and negative for annexin V. After treatment for 48 h, except for compounds 2, 5, 6, and 8, the other four compounds significantly induced cancer cell apoptosis at concentrations of 10 and 30 μ M.

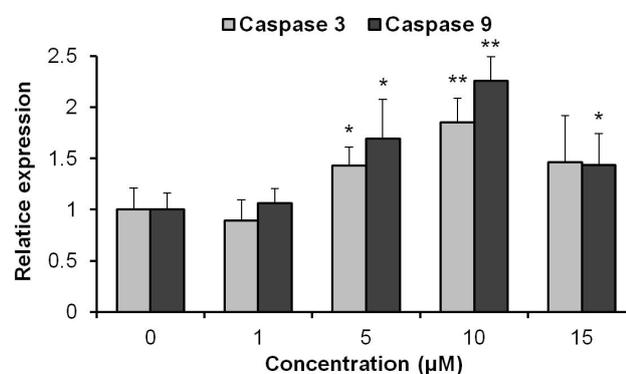
Compared to the control (5.1%), the percentages of cells in both early and late apoptosis for each compound (treatment with 10 μ M and 30 μ M) were: Compound 1 (47.2%, 68.0%), compound 3 (38.9%, 83.2%), compound 4 (86.4%, 88.8%), and compound 7 (60.6%, 73.9%), respectively. This result suggests that the novel compound 4 possesses the most potent apoptotic induction activity.

ACTIVITIES OF COMPOUND 4 ON CASPASES 3 AND 9

Apoptosis induction involves the activation of caspases, which can be divided into 2 groups: initiator caspases and their downstream effector caspases^[22]. Initiator caspases, such as caspase-8 and -9 are self-activated in response to apoptotic stimulations. Caspase-8 has been identified as the initiator caspase induced by death receptors, and caspase-9 has been proposed as another initiator caspase in the mitochondria-dependent apoptotic pathway. Caspase-3 is a critical effector caspase and initiates the apoptotic damage^[23]. Activation of caspase-3 requires the activation of initiator caspases, such as caspase-8 and caspase-9.

Furthermore, caspase 3 and caspase 9 are two known key proteins of the caspase family of proteases that are highly conserved in multicellular organisms and function as central regulators of apoptosis. They have been identified as playing a key role in the progression of apoptosis^[24-26]. To further characterize the potential mechanism of the anticancer effect of compound 4, we conducted caspase assays. The treatment of SW-480 cells with 5 μM of compound 4 for 24 h increased the protease activities of caspases 3 and 9 as shown in Figure 6. These activities were further increased when treated with 10 μM of compound 4. At this treatment concentration, protease activity was increased by 85.2% for caspase 3, and 125.5% for caspase 9. However, no increase of their activities was found at the treatment with 15 μM . The Western blot assay supported our results obtained by the protease activity assay.

Apoptosis is considered an important mechanism in the inhibition of cancer cells, and many cancer chemotherapeutic agents are



Figures 6 Effect of compound 4 on caspase 3 and 9.

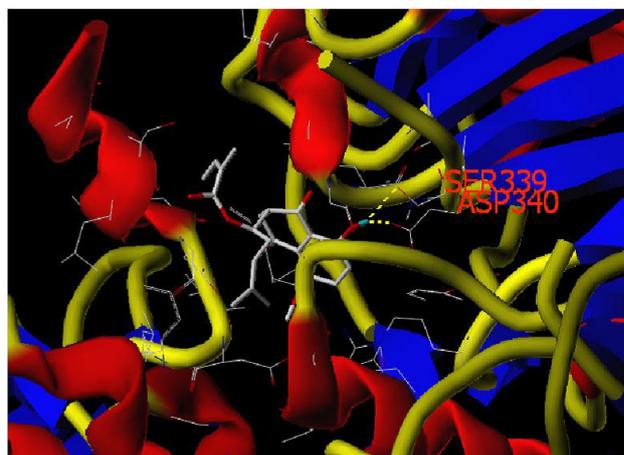
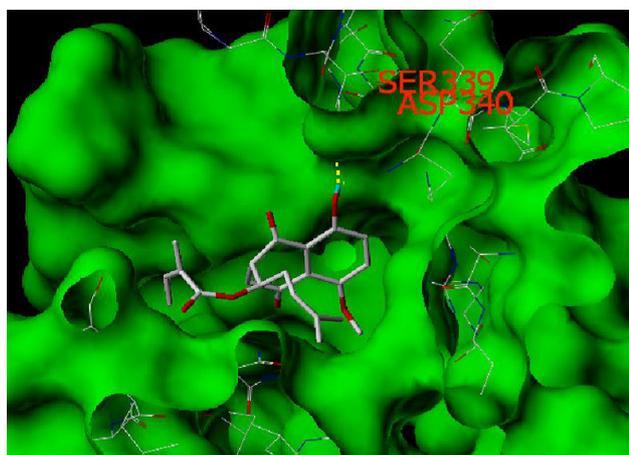
strong apoptotic inducers against cancer cells^[27]. In this study, we assayed the effect of eight compounds on cancer cell apoptosis, and observed that the anti-proliferation of potent compounds was related to their apoptotic response on colon cancer cells. To explore apoptotic induction mechanisms, we performed a caspase assay. We observed that compound 4 upregulated the expression of caspases 3 and 9, though this compound more significantly induced expression of caspase 9. Apoptosis occurs via a complex signaling cascade that is regulated at multiple points and involves many proteins. Caspase 9 is situated at critical points in apoptotic pathways and as a result plays a central role in apoptosis signal transduction^[28]. It is known that clinical drug development is a very long process and its success rate is low. However, many available anti-cancer drugs were originated from natural products. Compound 4 could be a good chemopreventive potential with possible clinical implications since the compound inhibited human colon cancer growth although our data are only an initial step for possible chemopreventive drug development.

RECEPTOR DOCKING ANALYSIS

The possible binding modes of compound 4 at the catalytic domains of human caspase 9 were predicted using the docking program Surflex-Dock. The structure of compound 4 was generated, and the protein crystal structure of caspase 9 was obtained. For the docking analysis, the protein structure was prepared by adding hydrogen atoms and missing side chain atoms and removing water molecules. Intermolecular interaction between compound 4 and caspase 9 was analyzed, and the key pharmacophore in the ligand was identified^[28,29].

Since compound 4 significantly increased caspase 9 activity, to address if compound 4 might physically interact with caspase 9, we examined compound 4 docking for human caspase 9. The Surflex-Dock program was used to predict the binding sites of compound 4 to caspase 9. The energetically most favorable position for compound 4 interactions with caspase 9 is shown in Figure 7. With *in silico* modeling, it is suggested that compound 4 forms hydrogen bonds with Asp-340 and Ser-339 at the active site of caspase 9 through its hydroxyl groups. In addition, compound 4 is predicted to show significant binding affinity for caspase 9, suggesting that compound 4 directly interacts with caspase 9.

Our docking analysis suggested the existence of an interaction site between compound 4 and caspase 9. Compound 4-induced apoptosis may occur in part through direct interactions with this protein.



Figures 7 Docking analysis of compound 4 on caspase 9.

CONCLUSION

The use of herbal medicines has been on the rise in cancer patients^[30,31]. As an alternative adjuvant to cancer chemotherapy, herbal medicines may act by increasing the sensitivity of neoplastic cells to chemotherapy and by reducing toxic and adverse effects induced by chemotherapeutic agents, without interfering with their tumoricidal effects^[32,33]. It is known that botanicals have been a significant resource to several of the currently used efficacious chemotherapeutic agents^[34,35]. There needs to be a continued effort to tap this resource and explore botanical or herbal active components with novel, potent, and distinct anti-cancer actions^[36].

This review is a good example to select resources contained compounds having enantiomers because the naphthoquinone derivatives from *A. tinctoria* like alkannin is specific as S-configuration which is an enantiomer of shikonin which has R-configuration. Therefore, we will be able to select species for the purpose of use.

We isolated eight compounds from *A. tinctoria*, including three novel compounds. Among the eight compounds, six are alkannin derivatives, including compound 4. The anti-proliferative activities of the eight compounds on different human colorectal cancer cell lines were determined indicating that 5-methoxyangenyalkannin (4) showed the most potent anti-proliferative and apoptotic induction activities compared to alkannin (1) and its other derivatives. Receptor docking analysis suggests the existence of an interaction site between compound 4 and caspase 9 resulting that compound 4-induced apoptosis may occur in part through direct interactions with this protein.

CONFLICT OF INTEREST

All authors declared no potential conflicts of interest.

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