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Antioxidant activities of alkannin, shikonin and *Alkanna tinctoria* root extracts in oil substrates

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Abstract

Alkannin and shikonin (A/S) are potent pharmaceutical substances with a wide spectrum of biological properties and comprise the active ingredients of several pharmaceutical and cosmetic preparations, and are used as food colorants. From a structural point of view, A/S bear both the quinone (naphthoquinone) and the phenolic moiety. Several hydroquinone compounds have been studied for their antioxidant effects, but very little is known about alkannin and *Alkanna tinctoria* root extracts that contain several naphthoquinone derivatives (mainly alkannin esters). The antioxidant activity of A/S and their derivatives, bearing both the quinone and the hydroquinone moiety, was studied in this paper. Several oils and fats were used as oil substrates for the antioxidant assay, namely, lard, corn oil, olive oil and sunflower oil.

Dichloromethane extract of *A. tinctoria* roots, containing mainly alkannin esters, showed satisfactory antioxidant activity in lard. Shikonin and monomeric alkannin presented very good antioxidant activity in lard, but their polymerization resulted in partial loss of this activity. The combination of the dichloromethane extract of *A. tinctoria* roots with caffeic acid showed a synergistic effect.

The oxidation of olive oil was retarded when monomeric or polymeric shikonin was present. It can be concluded that A/S and their derivatives, used as active ingredients in pharmaceutical, cosmetic and food preparations, may simultaneously exert antioxidant effects in oil substrates in addition to wound healing, anti-inflammatory and antimicrobial properties. The antioxidant activity of alkannin is low in corn oil, while monomeric shikonin, combined with citric acid, presents a very moderate antioxidant activity in sunflower oil.

Keywords: Alkannin; Shikonin; Hydroxynaphthoquinones; Antioxidant activity; Oven test

1. Introduction

Oils and fats are susceptible to oxidation. Traditionally, chemically synthesized compounds, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are used as antioxidants in oil products. However, some of these compounds have been questioned for their safety (Bran, 1975; Whysner, Wang, Zang, Iatropoulos, & Williams, 1994). Therefore, the use of natural antioxidants is now becoming important. Plants produce a variety of antioxidants against molecular damage from reactive species and thus certain natural products could play a preventive role due to their antioxidant properties. This research is a continuation of our investigations toward the exploitation of bioactive natural products, with prospects of use in pharmaceutical and cosmetic preparations, for their antioxidant activity.

In the context of evaluating natural products for their antioxidant properties, monomeric and polymeric alkannin, shikonin (A/S, Fig. 1) and *Alkanna tinctoria* root extracts are now being studied for their antioxidant effect on several oils and animal fats. A/S, which are naturally occurring hydroxynaphthoquinones, are potent pharmaceutical substances with a wide spectrum of biological properties, namely wound healing, antibacterial, anti-inflammatory and anticancer effects (for review see: Papageorgiou, 1980; Papageorgiou, Assimopoulou, Couladouros, Hepworth, & Nicolaou, 1999). Since many

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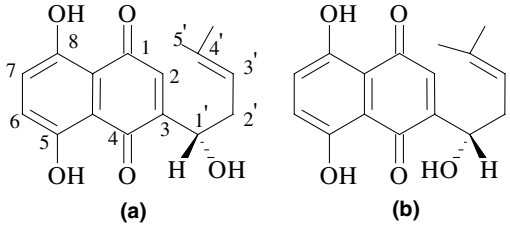

Fig. 1. The chiral pair alkannin and shikonin that possess major biological properties.

phenol and hydroquinone compounds have been found to exhibit antioxidative activities (Huang, Ho, & Lee, 1992), it seemed interesting to investigate the possible antioxidant activity in oil substrates of compounds having a hydroquinone and quinone structure, such as the lipophilic A/S and their derivatives in several forms (pure compounds, extracts) used in commercial pharmaceutical and cosmetic preparations.

It has been suggested that the wound healing activity of A/S may be due to their potent scavenging activity for superoxide anion radical, whereas anticancer and antimicrobial effects are due to A/S semiquinone radicals formation, which exhibit cytotoxicity via the generation of endogenous superoxide anion radicals (Sekine, Masumizu, Maitani, & Nagai, 1998). Recently (Gao, Kakuma, Oka, Sugino, & Sakurai, 2000), it was reported that shikonin interacts with reactive oxygen species ($\cdot O_2^-$, $^1O_2$, *tert*-butyl peroxyl radical (BuOO$\cdot$)) and has antioxidative ability against lipid peroxidation. Antioxidant activity of shikonin and its ester derivatives, isolated from *Lithospermum erythrorhizon* raw extract, was shown in lard, alone or in combination with Ve, BHT and citric acid (Weng et al., 2000). A/S and its parent moiety, naphthazarin, competed with DMSO for free OH$\cdot$ very efficiently and it was indirectly proposed that some of the claimed properties for A/S, such as anti-inflammatory and wound healing activity, may be attributed, at least partly, to their ability to interfere with free radical processes (Kourounakis, Assimopoulou, Papageorgiou, Gavalas, & Kourounakis, 2002).

The aim of this study was to examine the possible antioxidant activity of several natural products, such as alkannin, shikonin (A/S), *A. tinctoria* root extract and polymeric A/S in lard, which is a model oil substrate for antioxidant assay. Olive, corn and sunflower oils can be used as oily substrates in cosmetic and pharmaceutical preparations, which contain A/S and derivatives as active ingredients, and are also included in a great number of food preparations and thus the possible antioxidant effect of A/S on these oils has to be studied. The purpose of this study was to examine the effect of A/S on olive oil oxidation, since olive oil comprises the menstruum/dispersion medium for A/S and their derivatives in the commercial pharmaceutical preparation HELIX-DERM®, and can be used for extraction of A/S in several food preparations. This is the first time that polymerization of A/S is being studied for possible antioxidant activity.

2. Materials and methods

2.1. Chemicals

Lard was rendered from fresh pig fat, purchased from a local butcher's shop. Sunflower oil (Osolio) and corn oil (Corona) were purchased from a retail market. Virgin olive oil samples were provided by two olive processing plants, located in the areas of Chalkida and Chalkidiki (Greece). Caffeic acid (Sigma Chemicals, Steinheim, Germany) was used as a reference antioxidant substance. Citric acid (Sigma Chemicals, Steinheim, Germany) was used as metal chelator for sunflower oil and for its possible synergistic action with A/S. For the determination of peroxide values, acetic acid, chloroform, KI, starch and $Na_2S_2O_3$, all purchased from Merck, were used.

Alkannin and shikonin commercial samples were purchased from Roth (Karlsruhe, Germany) and Ikeda Corp. (Tokyo, Japan), respectively. These samples were thought to consist only of monomeric alkannin and shikonin, until, in a recent paper (Papageorgiou, Assimopoulou, & Kyriacou, 2002), we have proposed that they contain a great amount of polymers (specifically oligomers), mainly dimeric and tetrameric A/S. Thus, an alkannin commercial sample examined contained 17.4% monomeric, 13.8% dimeric and 68.8% tetrameric alkannin, while the percentages for a shikonin commercial sample were 45.7%, 23.9% and 30.4%, respectively. The possible antioxidant activity of A/S may depend on the degree of polymerization and, therefore, monomeric and polymeric alkannin and shikonin fractions were isolated.

Monomeric and polymeric alkannins were isolated by column chromatography from an alkannin commercial sample with various combinations of chloroform–methanol as eluants. Monomeric shikonin was isolated by column chromatography from a shikonin commercial sample. The purity of the isolated compounds was about 99%, as proved by HPLC and SEC techniques (Papageorgiou, Assimopoulou, & Kyriacou, 2002). Dichloromethane (DCM) extract of *A. tinctoria* roots was prepared from *A. tinctoria* roots (Wbag Resources, Zurich) using a Soxhlet apparatus (3 h). This extract contains mainly alkannin esters (both monomeric and polymeric) and, specifically, monomeric 37.1%, dimeric 6.4%, trimeric 6.5%, 15-meric 43.5% and 20-meric 6.4% (Assimopoulou & Papageorgiou, 2004). Extracts from *A. tinctoria* roots comprise a cheap and rich source of A/S derivatives, mainly esters.

2.2. Antioxidant assay

Oil oxidation was assessed by the oven test. Each oil sample (5 g) was transferred to a series of open transparent glass bottles. A specific concentration (%w/w oil) of each additive, tested for antioxidative activity, was added in a solution form, so that homogenization could be achieved and the solvent was afterwards removed. The solvent selected each time was either DCM, ethanol or methanol, depending on the solubility of each compound, in order to facilitate incorporation into the oil as a solution. Caffeic acid was diluted in absolute ethanol. A control sample was prepared under the same conditions without adding any additives. At least five replicate samples per tested preparation were stored.

The rate of oil oxidation was monitored by the increase of peroxide values (PV). 1 ± 0.1 g of each oil sample was weighed and subjected to iodometric determination (AOCS, 1990). The analyses were performed in duplicate. When the differences between the replicates were more significant (analysis with the student's t-test using significance level of $P < 0.05$), the measurements were repeated; however, such cases were exceptionally rare. Standard deviations, in all cases, were in the range of 3–10% from the mean. Values in tables represent means of two determinations. Oven temperature used was 45 and 65 °C, in order to achieve accelerative oxidation.

In this paper, four experimental series were designed. The combination of the tested preparations for each oil is analytically presented for each series of experiments in the respective Tables 1–6. In the first group of experiments, lard was used as the oil substrate (Tables 1 and 2). In this series, dichloromethane extracts of *A. tinctoria* roots (at different concentrations), pure monomeric and polymeric alkannin were studied for their possible antioxidant activities in lard (at 45 °C). Comparison was made with caffeic acid, while the synergistic effect of caffeic acid with DCM extract of *A. tinctoria* roots was examined (Table 1). Monomeric shikonin (the enantiomer of alkannin) and the polymeric one were tested for possible antioxidant activities in lard at 65 °C, in order to examine the influence of A/S chirality and degree of shikonin polymerization on antioxidant activity.

In the second group of experiments (Tables 3 and 4), two virgin olive oil samples were used as oil substrates. Monomeric and polymeric shikonin were tested for possible antioxidant activities in two olive oil samples at 45 and 65 °C. In the third group of experiments, corn oil was used as oil substrate for antioxidant assay of monomeric alkannin (Table 5) while, in the fourth group of experiments, sunflower oil was used as oil substrate in which shikonin, together with citric acid, were tested for their possible antioxidant activities. In most cases, citric acid was added with the tested compounds in sunflower oil as metal chelator (Table 6).

In each of the above experiments, several concentrations of the tested preparations were added in each oil, and selected according to typical concentrations of added compounds for each oil, e.g., 0.02 %w/w of pure compounds were added to lard, olive oil and sunflower oil and at least twice this for extracts, fractions or mixtures of compounds, while 0.05 %w/w of pure compounds were added to corn oil.

Table 1
Peroxide values of lard with and without additives (oven test, 45 °C)

Days/PV	$t = 0$	10	18	26	33	53	62	75
Lard (control)	10	189	205	278	305	–	–	–
Lard + DCM extract of *A. tinctoria* roots (0.04 %w/w)	–	17	26	182	260	–	–	–
Lard + DCM extract of *A. tinctoria* roots (0.02 %w/w)	–	19	37	268	283	–	–	–
Lard + caffeic acid (0.04 %w/w)	–	18	19	19	20	21	21	22
Lard + polymeric alkannin (0.04 %w/w)	–	72	271	273	275	–	–	–
Lard + DCM extract of *A. tinctoria* roots (0.02 %w/w) + caffeic acid (0.02 %w/w)	–	10	11	11	11	11	12	13
Lard + monomeric alkannin (0.04 %w/w)	–	14	18	18	18	18	20	–

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

Table 2
Peroxide values of lard with and without additives (oven test, 65 °C)

Hours/PV	$t = 0$	22	44	68	88	138
Lard	12	93	150	446	510	604
Lard + shikonin Ikeda (0.02 %w/w)	–	14	115	40	174	309
Lard + polymeric A/S (0.02 %w/w)	–	13	14	88	230	450
Lard + caffeic acid (0.02 %w/w)	–	13	14	25	50	71

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

Table 3
Antioxidant activity (PV) of monomeric shikonin in virgin olive oil (origin Chalkidiki; oven test, 45 °C)

Days/PV	0	4	10	19	37	52	76	92	122	172	212
Olive oil	9	10	13	15	17	18	28	41	52	300	607
Olive oil + monomeric shikonin (0.02 %w/w)	9	12	12	13	14	18	25	37	43	53	64

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

Table 4
Antioxidant activity (PV) of monomeric shikonin in virgin olive oil (origin Chalkida; oven test, 65 °C)

Hours/PV	0	139	237	308	360	408
Olive oil	9	21	24	39	50	89
Olive oil + polymeric shikonin (0.02 %w/w)	9	19	25	31	41	51
Olive oil + monomeric shikonin (0.02 %w/w)	9	18	23	30	39	46

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

Table 5
Antioxidant activity of monomeric alkannin in corn oil (45 °C)

Days/PV	0	2	4	6	10	14	19	25	32	37	46	52
Corn oil	1	2	3	2	3	4	4	5	13	23	48	70
Corn oil + monomeric alkannin (0.05 %w/w)	1	2	2	2	2	3	3	4	6	12	35	53

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

Table 6
Antioxidant activity of shikonin and citric acid in sunflower oil (65 °C)

Hours/PV	0	20	42	94
Sunflower oil	3	25	39	100
Sunflower oil + shikonin (0.02 %w/w) + citric acid (0.02 %w/w)	3	14	28	77

Values represent mean (PV) ($n = 2$). In all cases relative error was lower than 10%.

3. Results and discussion

In the present research, four groups of experiments were designed for testing the antioxidant activities of A/S (pure compounds, monomeric and polymeric) and extracts of *A. tinctoria* roots. In the first group, lard was used as substrate (Tables 1 and 2), and in the second one, virgin olive oil (Tables 3 and 4). Corn oil (Table 5) and sunflower oil (Table 6) were used as oil substrates in the third and fourth group of experiments, respectively.

The antioxidant activities of alkannin, shikonin, both monomeric and polymeric, A/S derivatives and extracts of *A. tinctoria* roots containing mainly A/S esters, were studied in four different oil substrates as reported (lard, olive oil, corn oil and sunflower oil), as presented in Tables 1–6. The possible antioxidant activities of monomeric alkannin, polymeric alkannin, and dichloromethane extract of *A. tinctoria* roots, compared to caffeic acid, were initially studied in lard at 45 °C (Table 1). The accelerative antioxidant test was also performed in lard at 65 °C for polymeric alkannin and monomeric shikonin (Table 2).

As shown, both 0.02 and 0.04 %w/w DCM extracts of *A. tinctoria* roots, that contain mainly A/S esters, reduce the oxidation rate of lard at 45 °C (0.04% extract presented the highest antioxidant activity). Monomeric alkannin had a very good antioxidant effect on lard, equal to that of caffeic acid, while polymeric alkannin was less active. This means that polymerization of alkannin reduces its antioxidant activity. Hence, the antioxidant activity of dichloromethane extract of *A. tinctoria* roots may be attributed to monomeric A/S derivatives (esters). A mixture of dichloromethane extract (0.02 %w/w with caffeic acid 0.02 %w/w) showed very high antioxidant activity, indicating a synergistic effect. Thus, dichloromethane extract of *A. tinctoria* roots can be used as a cheap and rich source of A/S derivatives with antioxidant properties. Probably, higher concentrations of dichloromethane extract of *A. tinctoria* roots, added to oils, will result in higher antioxidant activity.

From the comparison of monomeric shikonin and polymeric alkannin in lard at 65 °C, it can be concluded that only monomeric shikonin presented a significant antioxidant activity, while polymeric alkannin had a reduced antioxidant effect. Thus, in lard polymerization of A/S results in decreased antioxidant activity.

Olive oil is the substrate and the extraction medium for A/S and their derivatives during the preparation of

several pharmaceutical preparations (such as HELIXDERM®) and is included in several food preparations that can contain A/S derivatives. Therefore, the possible antioxidant activity of A/S in olive oil had to be tested. A good antioxidant activity of monomeric shikonin in olive oil was observed at 65 °C (Table 4) and at 45 °C (Table 3), while polymeric shikonin showed slightly decreased activity. The above data indicate that these active ingredients also enhance the stability of olive oil in commercial pharmaceutical preparations (such as HELIXDERM®) and also food and cosmetic preparations containing A/S besides their multiple biological activities.

Monomeric alkannin had a slight antioxidant effect on corn oil, as shown in Table 5. The induction period (PV 40) was increased from 35 to 40 days. In sunflower oil, monomeric shikonin (0.02 %w/w) was added with citric acid (0.02 %w/w), which is a Fe chelator (Weng et al., 2000; Hras, Hadolin, Knez, & Bauman, 2000). Shikonin, combined with citric acid, presented a very moderate antioxidant activity to sunflower oil at 65 °C (Table 6). The induction period (PV 70) was increased from 65 to 90 h.

As shown, virgin olive oil samples have significant stability, even without the addition of A/S derivatives, probably due to the presence of phonolic antioxidants including α-tocopherol, but A/S derivatives additionally improve the stability of olive oil, even after the influence of the antioxidant tocopherols is terminated. For example, the induction periods of olive and corn oils, to reach peroxide value 70, were 135 and 52 days, respectively (at 45 °C), whereas they were 380 and 65 h for olive oil and sunflower oil, respectively, at 65 °C. It therefore appears that olive oil is a better medium for the extract of A/S from *A. tinctoria* roots and the dispersion of alkannin and shikonin.

4. Conclusions

Monomeric alkannin and shikonin exhibit significant antioxidant activities in lard, which was used as a model oil substrate for antioxidant assay. Dichloromethane extract of *A. tinctoria* roots, that mainly consists of A/S esters, significantly reduced lard oxidation rate, while polymerization of A/S resulted in decreased ability compared to monomeric A/S. The antioxidant activity of A/S is clearly illustrated by its addition to lard.

The possible antioxidant effects of A/S and their derivatives were studied in vegetable oils, such as olive, corn and sunflower oils that can be used as oil base and extraction medium for A/S in cosmetic, pharmaceutical and food preparations containing A/S as active ingredients. The effect of A/S was examined on the stability of olive oil, which is used as the extraction medium or lipophilic base for several pharmaceutical preparations containing A/S as the active ingredients (such as HELIXDERM®). As proved, monomeric alkannin and shikonin presented significant antioxidant activities in two virgin olive oil samples tested. The antioxidant activity of A/S was shown to be slight in corn oil and moderate in sunflower oil. Polymerization of A/S reduced the antioxidant activity of A/S in each oil and fat tested. It was shown that monomeric alkannin and shikonin presented a stronger effect in olive oil, than in corn and sunflower oil, though olive oil is the most stable oil among those tested. It therefore appears that olive oil is a better oil base/extraction medium for these very important active natural ingredients.

A/S and their derivatives are natural products, used for colouring of wines and oils and as active ingredients in pharmaceuticals and cosmetics. The antioxidant activity of A/S was shown in oils, which creates a strong impetus for expanding the investigation of natural constituents responsible for the protection of oil against oxidation and the stability of lipophilic pharmaceutical preparations. It is very interesting and rare that A/S and derivatives exert so many biological properties, such as antioxidant, wound healing, anti-inflammatory, antimicrobial and anticancer activities and can be additionally used as natural antioxidants in cosmetics and pharmaceutical preparations, in which they are used mainly for their biological properties.

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