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The Antioxidant Activity of the Essential Oils of *Artemisia afra*, *Artemisia abyssinica* and *Juniperus procera*

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The essential oils of *Artemisia afra* Jacq., *Artemisia abyssinica* Schultz-Bip. and *Juniperus procera* Hoechst ex Endl. were examined for their potential radical scavenging activities. First a rapid evaluation of antioxidants was made using a TLC screening method. The abilities of the volatile oils to act as nonspecific donors for hydrogen atoms or electrons were checked in the diphenylpicrylhydrazyl assay. Oils from all three species showed positive results and were examined further. The oils of *A. afra* and *J. procera* were also effective hydroxyl radical scavenging agents when assessed in the deoxyribose degradation assay, whilst oils from *A. abyssinica* exhibited a paradoxical effect. In the *in vitro* assay for non-enzymatic lipid peroxidation in liposomes, the oils of *A. afra* and *J. procera* also displayed antioxidant potential. It was not possible to measure the effect of *A. abyssinica* oil in this system because certain components, e.g. alk-2-enals, interfered with the assay. The compounds that contribute to the radical scavenging activities of *A. afra* and *J. procera* were identified and then assessed for their effects in the various test systems. Finally, the qualitative and quantitative compositions of the essential oils were studied by GC-MS.

Keywords: Artemisia afra; *Artemisia abyssinica*; *Juniperus procera*; essential oils; antioxidant activity; GC-MS-analysis.

INTRODUCTION

Artemisia afra Jacq. (family Compositae), which is also known as African wormwood, 'Wild Als', 'Lanyana' and 'Menlonyana', is one of the best known and widely used plants in South African popular medicine. These bushy shrubs, grow up to 1.5 m high and are indigenous to the mountainous regions extending from the South West Cape to Abyssinia. Various preparations such as infusions, decoctions, molasses and alcohol extracts of *A. afra* are used for the treatment of coughs, colds, chills, stomachache, dry dyspepsia, as a purgative and for the cure of smallpox and malaria (Buchbauer and Silbernagel 1989; Jansen, 1981). The herbaceous leaves of this plant contain between 0.3% and 1.4% v/w of a bluish-green essential oil, with a strong fragrance that is similar to that of the volatile oils of other *Artemisia* species like *A. vulgaris* and *A. absynthium*. Because of its fine delicate odour the crop also is used to prepare medicinal wines, as a scent and as a substitute for incense in churches, places of worship and peoples' homes (Buchbauer and Silbernagel, 1989).

Artemisia abyssinica Schultz-Bip., another representative of the Compositae family, is distributed in regions of Northern Africa and the Middle East. Decoctions of the fresh plant, which is known in Saudi Arabia as 'Aathir', are used for the treatment of diabetes mellitus (Mossa, 1985). In Ethiopia medicinal uses of the herb include constipation, cold and rheumatism (Jansen, 1981). Phytochemical studies have proved the presence of alkaloids, flavonoids, sterols, diterpenes, tannins and volatile oil (Mossa, 1985). The fresh crop has a disagreeable smell but the scent of the pale yellow essential oil is quite pleasant. *Juniperus procera* Hoechst ex Endl., the only Cupressaceae growing naturally in the Southern hemisphere (Adams, 1990), occurs in the mountainous regions and highlands of East Africa. The evergreen tree, that reaches a height of 30–45 m, has other common names in Ethiopia such as 'tid' (Amarinia, Tigrinia) and 'sareda' (Amarinia, Oromigna) (Jansen, 1981). Different preparations of the aerial parts of *J. procera*—like its fruits, resin, leaves, twigs and buds—are used for the treatment of ulcers, headaches, stomach disorders, intestinal worms, as an emmenagogue, to relieve rheumatic pains, to heal wounds and to cure liver diseases (Jansen, 1981). Furthermore the wood is used for buildings and also in the manufacture of pencils (African pencil cedar). The leaves contain between 0.6% to 1.2% v/w essential oil. The composition of this oil has been analysed (Adams, 1990) although no tests have been used to examine possible pharmacological activities.

Traditionally these African plants are used to treat various inflammatory diseases—like rheumatism, fever and diabetes—in which free radical oxidative stress has been implicated in the pathogenesis of these disorders (Halliwell and Gutteridge, 1989). Much of the damage

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inflicted on cells, biomembranes or DNA *in vivo* is thought to be due to the conversion of biologically relevant reactive oxygen species (ROS) into highly reactive oxidants (Halliwell, 1990), such as the hydroxyl radical, ˙OH.

In recent studies, *A. afra* oil demonstrated antifungal (Gundidizia, 1993) and antimicrobial (Graven *et al.*, 1992; Rabe and van Staden, 1997) activities and it was tested in the so called agar diffusion test (Graven *et al.*, 1992) for its possible antioxidant activity. Apart from these studies no other *in vitro* tests have been performed to determine the radical scavenging properties of *A. afra* or the volatile oils of *A. abyssinica* and *J. procera*.

In the present study, the effects of the three essential oils from *A. afra, A. abyssinica* and *J. procera* were examined as reducing agents and ˙OH radical scavengers using an assay for non-enzymatic lipid peroxidation in liposomes and the deoxyribose degradation test.

MATERIALS AND METHODS

Plant materials. *Artemisia afra* and *A. abyssinica* herbs were purchased in a local market in Addis Ababa in June 1998. *Juniperus procera* leaves were collected in June 1998 from a tree growing in the School of Pharmacy campus, Addis Ababa. Botanical identity was confirmed by Mr Melaku Wondafrash, the National Herbarium, Department of Biology, Faculty of Science, Addis Ababa University, Addis Ababa, Ethiopia. Voucher specimens were deposited at the Department of Pharmacognosy, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia.

Chemicals. Chemicals were obtained from Fluka (Buchs, Switzerland). Diphenylpicrylhydrazyl and bovine brain phospholipids were purchased from Sigma (Sigma Aldrich GmbH, Sternheim, Germany). Limonene and linalool were obtained from Roth (Karlsruhe, Germany) and *p*-cymene was purchased from the British Drug House Ltd (Poole, Great Britain). TLC was carried out on silica gel F_{254} aluminium sheets (Merck, Darmstadt, Germany).

Extraction of the oils. The volatile oils were obtained by hydrodistillation from the aerial parts of *A. afra, A. abyssinica* and *J. procera* using a Clevenger-type apparatus. Distillation was carried out on 500 g of each of the fresh plant materials for 6 h. The percentage yields (v/w) of the oils of *A. afra, A. abyssinica* and *J. procera* were 1.5%, 1.2% and 1.1%, respectively.

GC-MS-analysis. Qualitative and quantitative compositions of the oils were analysed by means of GC-MS. Analysis was carried out on a Hewlett-Packard 5890 Series II plus gas chromatograph interfaced to a Hewlett Packard 5989B mass spectrometer. Separations were performed on Ultra 1 (49 m × 0.20 mm I.D., 0.11 μm, Hewlett Packard) and DB-Wax (60 m × 0.25 mm I.D., 0.25 μm, J&W Scientific) capillary columns. The carrier gas was helium (1.0 mL/min constant flow). Oven temperature was programmed within a range 70–230 °C with a heating rate of 2 °C/min. Injector and interface temperatures were 230 °C and 250 °C, repectively. EI mass spectra were recorded at 70 eV ionization voltage. The oil samples (0.5 μL of 1:10 dilutions in hexane) were injected by split injection (1:30). A library search was carried out using the Wiley 138K registry of mass spectral data and the laboratory's own database.

Spectral data also were compared with reference compounds and the results confirmed with the aid of retention time indices from published sources (Bucar and Schweiger, 1998; Davies, 1989). Quantification of the relative amounts of the individual components was performed according to the Area Percent Method without consideration of calibration factors.

TLC SCREENING FOR ANTIOXIDANTS

Dilutions of the volatile oils (5 μL, 1:10 in hexane) were spotted onto silica gel sheets and developed in toluene–ethyl acetate (97:3 v/v). The plates were sprayed with a 0.2% solution of the stable radical, diphenylpicrylhydrazyl (DPPH) (Cuendet *et al.*, 1997; Kirby and Schmidt, 1997). Active compounds were detected as yellow spots on a purple background. Zones where the colour changed within 30 min (after spraying) were taken as positive results.

DPPH assay. This spectrophotometric assay uses DPPH as a reagent (Cuendet *et al.*, 1997; Kirby and Schmidt, 1997). Fifty μL of various concentrations of the volatile oils or the terpenes were added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm using a Jasco 7800 spectrophotometer. Tests were carried out in triplicate.

Deoxyribose degradation assay. This test was adopted from a method described by Halliwell *et al.* (1987). Solutions of the reagents were always prepared freshly. The reaction mixture contained in a final volume of 1.0 mL, 100 μL of 2-deoxy-2-ribose (28 mM in KH_2PO_4 K_2HPO_4 buffer, pH 7.4), 500 μL solutions of various concentrations of the tested oils or the pure compounds in buffer, 200 μL of 1.04 mM EDTA and 200 μM $FeCl_3$ (1:1 v/v), 100 μL of 1.0 mM H_2O_2 and 100 μL of 1.0 mM ascorbic acid. Test samples were kept at 37 °C for 1 h. The free radical damage imposed on the substrate, deoxyribose, was measured using the thiobarbituric acid test (Houghton *et al.*, 1995; Aruoma *et al.*, 1989). One mL of thiobarbituric acid, TBA (1%), and 1.0 mL trichloroacetic acid, TCA (2.8%), were added to the test tubes and were incubated at 100 °C for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Reactions were carried out in triplicate.

Inhibition (*I*) of deoxyribose degradation in percent was calculated in the following way:

$$I(\%) = 100 \times (A_0 - A_I/A_0)$$

where A_0 is the absorbance of the control reaction, and A_I is the absorbance of the test compound.

Assay for site specific actions. To measure possible site specific actions (Halliwell *et al.*, 1987; Aruoma, 1996; Aruoma *et al.*, 1997), i.e. whether the oils possess prooxidative activities, if they can stimulate an oxidative process or if they generate fragments, which react with

thiobarbituric acid, the described deoxyribose method was adopted in three ways:

1. $FeCl_3$ was used instead of a solution of Fe^{3+}-EDTA.
2. Ascorbic acid was omitted from the test system.
3. The reaction was performed without the sugar deoxyribose. The lacking volume was made up with phosphate buffer pH 7.4.

Assay for non-enzymatic lipid peroxidation in liposomes. The assay was performed as described by Houghton *et al.* (1995) with minor changes. The material for peroxidation was prepared from Type VII Folch bovine brain extract (Sigma). One mL of the reaction mixture contained 500 μL of the bovine brain extract (5 mg/mL), 300 μL of a solution containing different concentrations of the test compounds, 100 μL 1 mM $FeCl_3$ and 100 μL 1 mM ascorbic acid to induce $^{\cdot}OH$ radical generation. After an incubation period of 1 h at 37 °C the extent of lipid peroxidation was measured by the TBA-reaction. One mL TBA, 1.0 mL TCA and 100 μL butylhydroxytoluene were added and the test vials heated at 100 °C for 20 min. After cooling, 2.5 mL *n*-butanol was added and the samples centrifuged at 3500 rpm for 5 min. The absorbance was read at 532 nm. Calculations were performed as detailed above.

RESULTS AND DISCUSSION

GC-MS analysis

Forty-five compounds of *A. afra* oil were identified (89.1 area%). The main compounds were camphor (26.8%), davanone (16.6%), bornyl acetate (3.8%), 4-terpineol (3.6%) and chamazulene (3.2%).

Thirty-two substances (60.5%) of *A. abyssinica* were identified with 4-hydroxycyclohexanemethanol (21.3%) and α-terpinolene (9.2%) as the main components and 64 components of the essential oil of *J. procera* (81.9 area%), which contains monoterpenes such as α-pinene (8.4%), delta-3-carene (7.9%) and 4-terpineol (5.0%). The results for *J. procera* and *A. abyssinica*-oil corresponded with published data (Adams, 1990; Alkhathalan and Al Hazimi, 1996) but not for *A. afra*. This was not surprising since *A. afra* comprises a wide range of divergent genotypes and preliminary studies (Graven *et al.*, 1986; Moody *et al.*, 1994) have shown that the oil composition of individual plants may vary widely. Comparing our results with other data regarding the chemical composition of *Artemisia* oil, it became evident that the sesquiterpene chamazulene, which not only gives the oil its blue colour but also seems to be responsible for the radical scavenging effect of the oil, has not been mentioned as a component previously. It is possible that the analysed volatile oil was from a new chemotype of *A. afra* or that its chemical composition was influenced by extrinsic factors that caused major changes.

Rapid TLC screening

The first test for antioxidant activities was a rapid TLC screen. Chromatograms of the essential oils were sprayed with DPPH. In the case of *A. afra* only one zone, corresponding to chamazulene, changed colour from violet to yellow over time. *J. procera* oil produced four active spots (corresponding to α-pinene, *p*-cymene, linalool and limonene). On the TLC sheet with *A. abyssinica* oil only one spot appeared within 30 min. It was identified by means of reference substances as linalool. All five compounds were subjected to further testing.

DPPH assay

The abilities of the test compounds (both the volatile oils and the components which were detected in the TLC screening) to donate hydrogen atoms or electrons were measured spectrophotometrically. Each of the tested oils reduced DPPH to the yellow coloured product, diphenylpicrylhydrazine, and the absorbance at 517 nm declined. The strongest effect was measured for *A. afra* oil with an IC_{50} of 1.1 μL/mL. The IC_{50} of the second *Artemisia* oil was 28.9 μL/mL and it took 14.9 μL/mL of the *Juniperus* oil to produce a 50% inhibition. The individual compounds did not possess such strong reducing effects and it was not possible to measure concentrations for a 50% inhibition under these test conditions. Their activities in the TLC screening assay might be explained by synergistic effects. A wide range of monoterpenes have very similar retention indices, so it is quite reasonable that the separation on TLC was incomplete and that the active zones with DPPH spraying reagent reflected the presence of other substances. When the individual compounds were tested in this assay, they were unable to reduce DPPH.

Deoxyribose degradation

For this test $^{\cdot}OH$ radicals were generated by reaction of ferric-EDTA together with H_2O_2 and ascorbic acid to attack the substrate deoxyribose. The resulting products of the radical attack form a pink chromogen when heated with TBA in acid solution (Halliwell *et al.*, 1987; Aruoma *et al.*, 1989). When the oils or the pure substances were incubated with this reaction mixture they were able to interfere with the free radical reaction and could prevent damage to the sugar. The results are shown in Fig. 1, concentrations for 50% inhibition were 0.46 μL/mL for *A. afra* and 0.40 μL/mL for *J. procera*. In the case of *A. abyssinica*, first a radical scavenging effect occurred but when reaction mixtures contained higher concentrations of the volatile oil the absorbance increased and the antioxidant effect declined. At a certain concentration a pro-oxidative effect occurred (additionally) and the absorbance at 532 nm increased. It was shown that certain compounds in the oil, like alkanals and other aliphatic aldehydes, reacted with the reagent TBA and formed coloured products. This paradoxical effect could be seen more clearly in the experiments for site specific actions and the assay for non-enzymatic lipid peroxidation. Concentrations for 50% inhibition of the pure compounds (IC_{50}) are listed in Table 1.

Assay for site specific actions

The deoxyribose assay was modified in three different

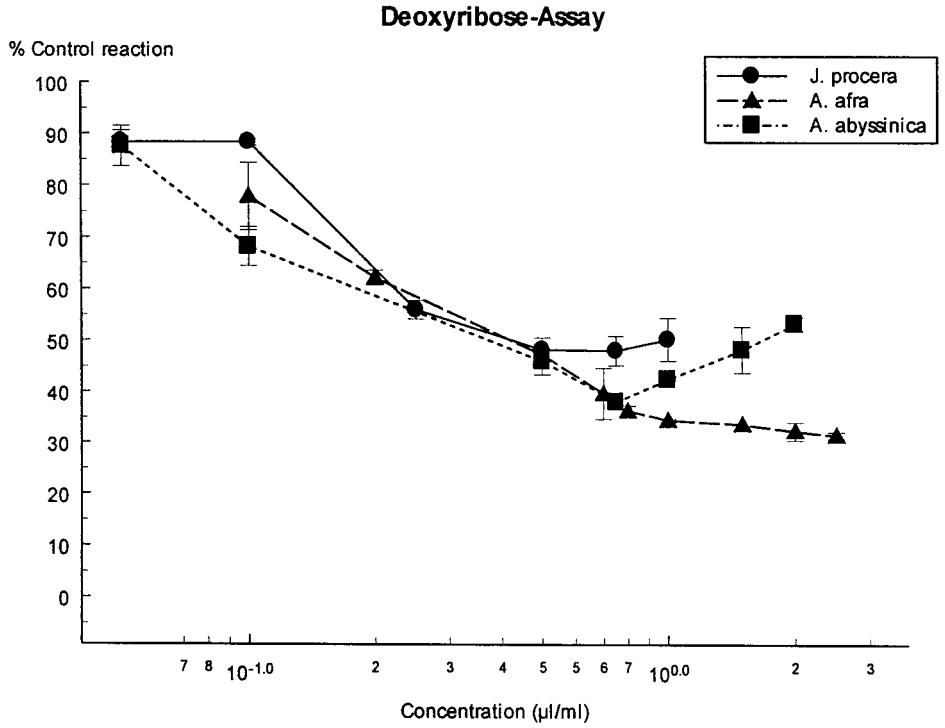

Figure 1. Effects of the essential oils of *Juniperus procera, Artemisia afra* and *Artemisia abyssinica* on the radical degradation of 2-deoxy-2-ribose.

ways to assess whether the oils exhibited site specific effects.

For the first test, EDTA was omitted from the reaction mixture, iron was added as ferric chloride, not in complexed form. Some of the Fe^{3+} ions bind directly to the sugar and its degradation becomes site specific. The formed hydroxyl radicals attack deoxyribose immediately (Aruoma *et al.*, 1987). An inhibition of this degradation in the absence of EDTA depends not only on a scavenger's ability to react with ·OH but also on its potential to form complexes with iron ions. *A. afra* oil (0.53 µL/mL) could reduce the deoxyribose degradation to 75% of the value of the control reaction. *J. procera* oil was not able to inhibit this reaction while *A. abyssinica* seemed to have a stimulating effect on it.

Table 1. Concentrations for a 50% inhibition of the tested monoterpenes and the sesquiterpene chamazulene in the deoxyribose degradation assay and the test for non-enzymatic lipid peroxidation

Compound	IC_{50} in the deoxyribose degradation test (µL/mL)	IC_{50} in the assay for nonenzymatic lipid peroxidation (µL/mL)
α-Pinene	0.78	0.51
Chamazulene	0.042	0.0021
p-Cymene	0.91	0.69
Limonene	1.05	1.14
Linalool	0.28	0.67

To measure potential pro-oxidative effects the test was done without ascorbic acid and a slower rate of ·OH radical generation occurred. Pro-oxidative substances stimulate the radical reaction, they induce damage to the substrate and the absorbance at 532 nm increases significantly. While *A. afra* and the Cupressaceae oil did not show such effects, the presence of *A. abyssinica* induced the radical process and the absorbance increased.

After checking the change of absorbance of the mixture containing *A. abyssinica* oil at different wavelengths, it was obvious that the maximum of extinction of the formed chromogen was at 455 nm. The increase of absorbance at 532 nm (where the maximum absorbance of the TBA-complex can be found) was recorded only as a side effect. The colour of the reaction mixture was also different from the normal TBA reaction mixture. At the start of the experiment, the solutions were yellow instead of pink, about half an hour later they turned into orange and finally (some hours later) the reaction mixtures were red.

Kosugi and colleagues (1987) discovered that alkanals, alk-2-enals and alka-2,4-dienals produce yellow, orange and red absorbing products, respectively, in the presence of TBA at low pH. While yellow chromogens are generated soon after mixing the aldehydes with TBA, the red pigments appear about 6 h after the start of the reaction.

When checking the qualitative composition of *A. abyssinica* oil, aldehydes such as 2-butenal, 2-hexen-1-al or octadecenal were found. This might explain the stimulating effect of the deoxyribose degradation under site specific conditions.

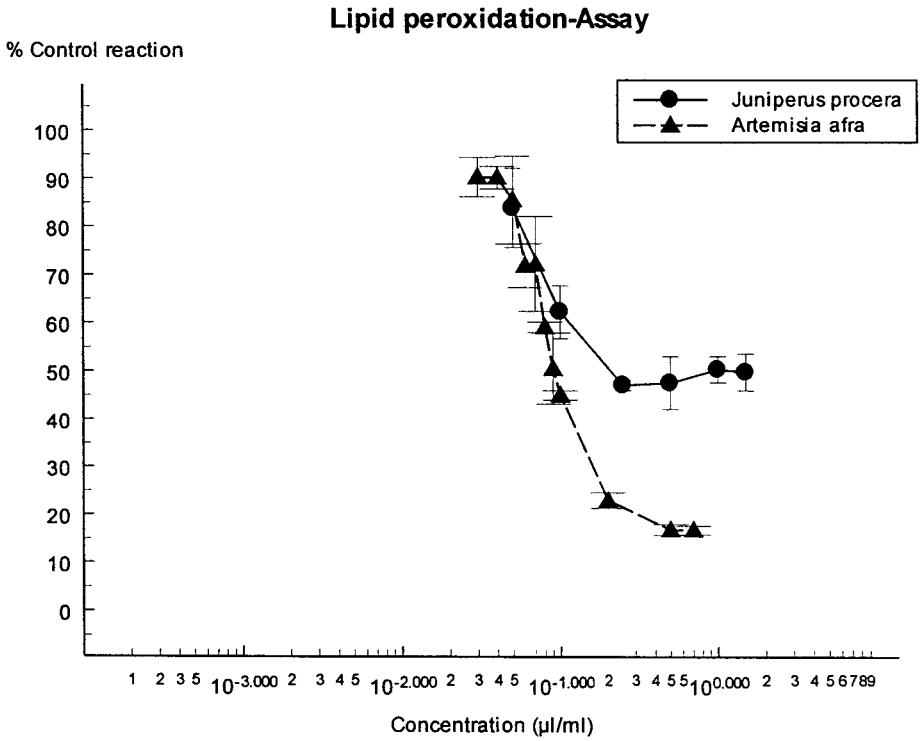

Figure 2. Inhibition of non-enzymatic lipid peroxidation by the essential oils of *Juniperus procera* and *Artemisia afra*.

To confirm this, various aldehydes such as trans-2-hexen-1-al, were tested. All of them increased the absorbance at 455nm in the beginning and a few hours later at 532 nm.

The extent of contribution of such alkenals to the pink pigment formation in the TBA test is not known. Compared with the reaction of malondialdehyde, MDA (which in recent years was thought to be the only component to react with TBA but today it is known that this presumption was wrong; Saari Csallany *et al.*, 1984), the coloration is only weak and can only be seen when the main reaction is suppressed (Kosugi *et al.*, 1987).

The formation of these disturbing complexes can be slightly reduced when reaction mixtures and TBA are incubated at lower temperatures (at 80 °C the reaction was reduced to 66% compared with the reaction level at 100 °C), but it can only be abandoned when molecular oxygen is removed from the reaction mixture. The mechanism of the formation is not known. The case of *A. abyssinica* oil demonstrates the importance of checking (even) small details in the assay such as the look and the colour of the test solution.

Finally, it was tested to see if the essential oils themselves could form oxidation-products that react with TBA to give coloured products. By omitting the sugar deoxyribose from the reaction mixture the volatile oils were the only substrates to react with ˙OH radicals and to form thiobarbituric acid reactive material, TBARs (Aruoma and Cuppett, 1997).

A. afra and *J. procera* did not show such effects. In the case of *A. abyssinica*, the absorbance was higher than that measured for the control reaction. This was probably the result of the reaction of the aldehydes in the oil and not the result of TBARs generation.

Non-enzymatic lipid peroxidation

In this assay peroxidation of bovine phospholipid liposomes is induced by $Fe(III)Cl_3$ and ascorbic acid as reducing agents. Hydroxyl radicals are generated by mixing Fe^{3+} and ascorbate and they attack the biological material. This leads to the formation of MDA and other aldehydes, which form a pink chromogen with TBA, absorbing at 532 nm.

As demonstrated in Fig. 2, the oils of *A. afra* and *J. procera* inhibit lipid damage caused by hydroxyl radicals. The IC_{50} were 0.09 µL/mL and 0.20 µL/mL, respectively. Values for a 50% inhibition caused by the active main compounds of these two oils are shown in Table 1.

It was not possible to measure the effect of *A. abyssinica* oil in the non-enzymatic lipid peroxidation because the test was disturbed by the reaction of some of the oil's components with TBA. Again the absorbance at 455 nm increased significantly and as a consequence the exact absorbance at 532 nm could not be read.

The antioxidant effect of *A. afra* may be due to its chamazulene content, while in the Cupressaceae oil mainly monoterpenes act as radical scavenging agents.

It seems possible that the observed radical scavenging properties of the essential oils of *Artemisia afra* and *Juniperus procera* might contribute to the positive effects of these two African plants in the treatment of certain inflammatory diseases.

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