

Inhibition of the Initiation Stage of Carcinogenesis by *Salvia disermas* Constituents

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Phytochemical studies of an ethanolic extract of the aerial parts of *Salvia disermas* resulted in the isolation of seven known compounds, rosmarinic (**1**) and caffeic (**2**) acids, salvigenin (**3**), luteolin (**4**), luteolin 7-*O*- β -arabinoside (**5**), luteolin 7-*O*- β -glucoside (**6**), and ocotillol II (**7**). The initiation stage of carcinogenesis is triggered by activation of procarcinogens by phase I enzymes, such as cytochrome P-450 1A, and oxidative stress that leads to DNA damage. The initiation stage is countered by phase II detoxification enzymes such as glutathione S-transferases (GST), quinone reductase (QR), epoxide hydrolase (mEH) besides conjugation with thiols. We aimed to investigate the cancer chemopreventive and tumour anti-initiating activity of the ethanolic extract of the aerial parts of *Salvia disermas* and its constituents. The *S. disermas* extract was a promising inhibitor of CYP1A activity, inducer of GST, QR, and mEH activities, enhancer of thiol content, radical scavenger, and inhibitor of DNA damage. On the other hand, **3** was an enhancer of thiol content and QR activity, while **4** was an inhibitor of CYP1A activity, inducer of QR activity, and radical scavenger of ROO[•], and **5** was an inducer of GST activity and inhibitor of DNA damage. The present study indicated that the ethanolic extract of *S. disermas* and **4** are promising anti-initiating and multipotent blocking agents.

Key words: Tumour Anti-Initiating, *Salvia disermas*, Flavonoids

Introduction

Salvia constitutes almost 25% of the Lamiaceae and is the largest genus of this family. In South Africa, the majority of *Salvia* species is distributed predominantly in the Cape region. *Salvia* species are used in many regions of the world to treat various diseases. *Salvia disermas* Linnaeus is commonly found in South Africa and was determined to have varying pharmacological activities including antimicrobial, antioxidant, anti-inflammatory, antiplasmodial, cytotoxic and antituberculosis. The oil of *S. disermas* is dominated by linalyl acetate (34.5%) and contains other compounds such as shyobunone (10.7%) and epi-isoshyobunone (6.2%), which have not been detected in other

species. Salvigenin and oleanolic acid/ursolic acid are abundant in *S. disermas* (Kamatou *et al.*, 2006a, b, c).

Epithelial carcinogenesis is a multistep process in which accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and, finally, carcinoma (Tsao *et al.*, 2004). It affects different stages of carcinogenesis including initiation, promotion, and progression. Initiation involves direct DNA binding and damage by carcinogens; it is rapid and irreversible. Promotion, which involves epigenetic mechanisms, leads to pre-malignancy; it is generally irreversible. Progression, which is due to genetic mechanisms, is the period between pre-malignancy and the oc-

currence of cancer; it is also generally irreversible. With rare exceptions, the stages of promotion and progression usually span decades after the initial carcinogenic exposure (Tsao *et al.*, 2004).

In order to exert their neoplastic actions in the initiation stage of carcinogenesis, most chemical carcinogens must be bioactivated, usually by conversion to electrophilically reactive metabolites (Autrup, 1990). In most instances, the formation of reactive metabolites from procarcinogens is catalyzed by microsomal and nuclear monooxygenase enzyme systems consisting of NADPH-cytochrome P450 (NADPH-CYP) reductase and isozymes of CYP (Tsao *et al.*, 2004). Once formed, reactive electrophiles may be detoxicated enzymatically either by conjugation with reduced glutathione (GSH), a reaction catalyzed by glutathione S-transferases (GST), or, if the metabolite is an epoxide, by hydration to the corresponding *trans*-dihydrodiol under the influence of epoxide hydrolase (mEH) (Tsao *et al.*, 2004). Dihydrodiols generated from certain polycyclic aromatic hydrocarbons can be metabolized further by polycyclic aromatic hydrocarbon-induced isozymes of CYP to yield extremely reactive and carcinogenic dihydrodiol epoxides (Cannady *et al.*, 2002).

In the present work, we describe the isolation and structure elucidation of the phenolic and terpenoid compounds from an ethanolic extract of the aerial parts of *S. disermas*. We also evaluate the cancer chemopreventive activity of the ex-

tract, fractions and isolated compounds, with special focus on their ability to prevent the initiation stage of carcinogenesis.

Results and Discussion

Phytochemical fractionation of an ethanolic extract of fresh aerial parts of *S. disermas* resulted in the isolation of seven known compounds (Fig. 1), rosmarinic (1) (Petersen and Simmonds, 2003) and caffeic (2) (Dusut *et al.*, 2001) acids, salvigenin (3) (Talapatra *et al.*, 1974), luteolin (4) (Mabry *et al.*, 1970), luteolin 7-*O*- β -arabinoside (5) (Shindo *et al.*, 2008), luteolin 7-*O*- β -glucoside (6) (Mizuo *et al.*, 1987), and ocotillol II (7) (Akihisa *et al.*, 1998). The compounds were isolated for the first time from *S. disermas*, and identified based on ^1H and ^{13}C NMR, UV, and MS data.

Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or pre-neoplastic tissue (Hong and Sporn, 1997). Most chemical carcinogens require metabolic activation by phase I enzymes in order to induce a biological response. Induction of phase II drug-metabolizing enzymes such as GST, quinine reductase (QR) or mEH is considered a major mechanism of protection against chemical stress and initiation of carcinogenesis (Bertram, 2000). The production of reactive metabolites is largely dependent on the primary metabolism by cytochrome P450 enzymes. The result of exposure

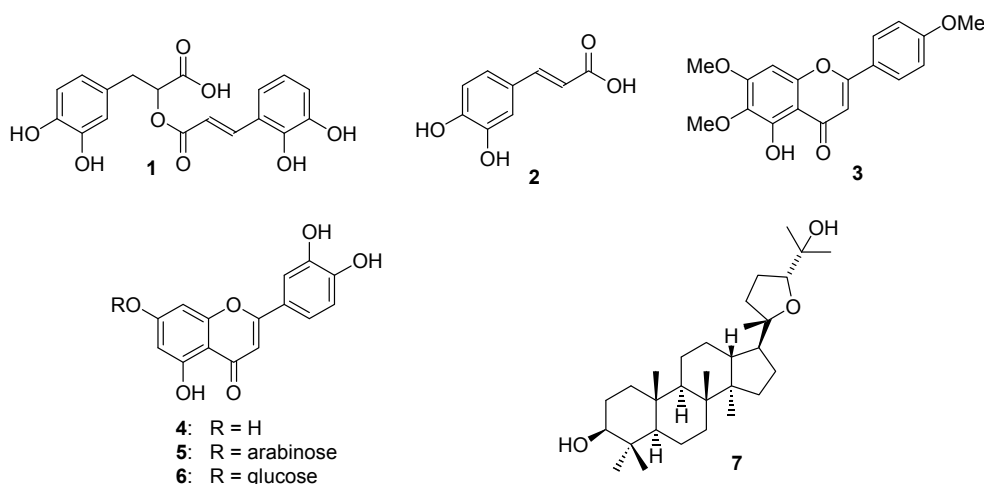


Fig. 1. Chemical structures of the compounds isolated from *S. disermas*.

to an environmental toxin in terms of acute or chronic toxicity largely depends on the balance between these two processes. We examined the effect of an *S. disermas* extract, fractions and compounds on the activity of cytochrome P450 1A (CYP1A), one of the cytochrome P450 isoenzymes, which is involved in the activation of procarcinogens to ultimate carcinogens. Our results indicated that the ethanolic extract of *S. disermas*, fraction III and 4 can be identified as potent inhibitors of CYP1A activity ($P < 0.05$) with an inhibition percentage of 44.02, 31.94, and 38.97%, respectively (Fig. 2), compared with the initial activity of β -naphthoflavone-stimulated cells. On the other hand, the rest of fractions and compounds possessed insignificant inhibition of CYP1A activity, $<15\%$ ($P > 0.05$), while 1 could not be assayed for CYP1A activity due to its high autofluorescence at the assay excitation wavelength of 408/20 nm and emission wavelength of 460/40 nm, which strongly interfered with the assay results.

A key determinant of the cellular response to oxidative stress relates to the level and form of GSH, which helps in destruction of hydrogen per-

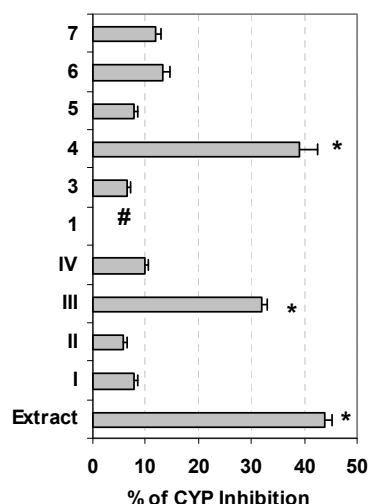


Fig. 2. The anti-initiating activity through modulation of the carcinogen metabolism: the inhibitory effect of 1 $\mu\text{g/ml}$ of each sample on CYP1A activity. The symbol # represents compounds that could not be assayed for CYP1A activity due to their high autofluorescence that interfered with the assay results at the excitation wavelength 408/20 nm and emission wavelength 460/40 nm. Data was expressed as mean \pm SD and control represents 100% activity.

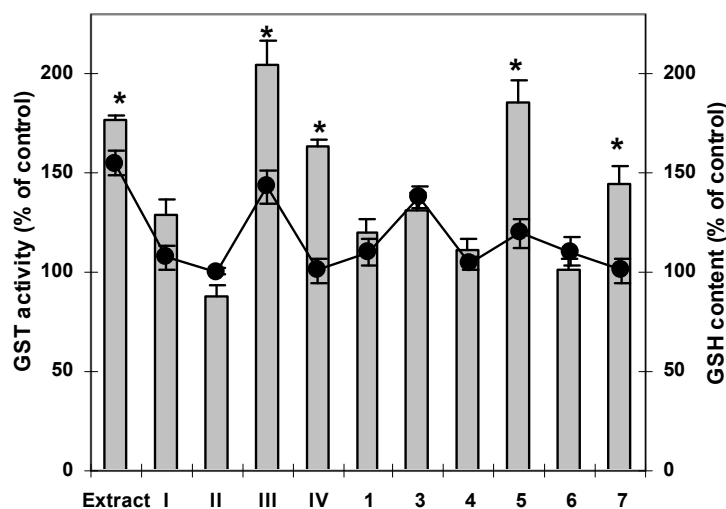


Fig. 3. The anti-initiating activity through modulation of the carcinogen metabolism: the effect of treatment with 10 $\mu\text{g/ml}$ of each sample for 48 h on the GST activity (bars) and the non-enzymatic antioxidant GSH content (circled line) in Hepa1c1c7 cells. Data is expressed as mean \pm SD and control represents 100% of the scale, which was 38.85 nmol/mg protein of GST and 26.39 nmol/mg protein of GSH.

oxide, lipid peroxides and free radicals (Aggarwal and Shishodia, 2006). A major factor that affects GSH homeostasis is its utilization by conjugation, primarily via GST, which are responsible for the detoxification of a wide range of substrates including xenobiotics, and occupational and environmental carcinogens such as pesticides and polycyclic aromatic hydrocarbons; GST evolve as a cellular protection system against toxins and carcinogens (Rooseboom *et al.*, 2004).

Conjugation and detoxification of active carcinogens is one of the important mechanisms to halt the tumour initiation step. The total GST were investigated in cultured Hepa1c1c7 cells as a representative for phase II enzymes. After 48 h of incubation with 10.0 $\mu\text{g/ml}$ of the extract, fractions and compounds, the results revealed that the total GST activity was significantly induced by the ethanolic extract of *S. disermas*, fractions III and IV, and compounds 5 and 7 ($P < 0.05$), while the rest of fractions and compounds led to an insignificant change in the enzyme activity ($P > 0.05$) (Fig. 3). The enhancement of the nonenzymatic antioxidants, total thiols, helps in counteracting the deleterious effect of reactive oxygen species (ROS). The treatment with 10.0 $\mu\text{g/ml}$ of the extract, fractions and compounds resulted in an insignificant change in the total thiol content, except for the extract, fraction III and 3, which led to a significantly elevated level of the cellular total thiol content (Fig. 3). Phase II enzymes generally conjugate activated xenobiotics to endogenous ligands. The QR activity, which is induced coordinately with other phase II enzymes like GST and contributes to the detoxification of quinones, was investigated in murine hepatoma cell culture. After a 48-h treatment, we found that most of the tested samples resulted in a variable degree of significant induction of QR activity ($P < 0.05$ to $P < 0.01$), except for fraction I and compounds 5, 6, and 7, which resulted in an insignificant increase of the enzyme activity ($P > 0.05$), as shown in Fig. 4A.

mEH is an important metabolic enzyme that catalyzes the addition of water to alkene epoxides and arene oxides. mEH has a wide substrate specificity and has the capacity to both bioactivate and detoxify xenobiotics including the epoxide metabolites of polycyclic aromatic hydrocarbons, 1,3-butadiene, benzene, and aflatoxin B1 (Cannady *et al.*, 2002). In the present work we measured the cellular mEH activity. Our findings indi-

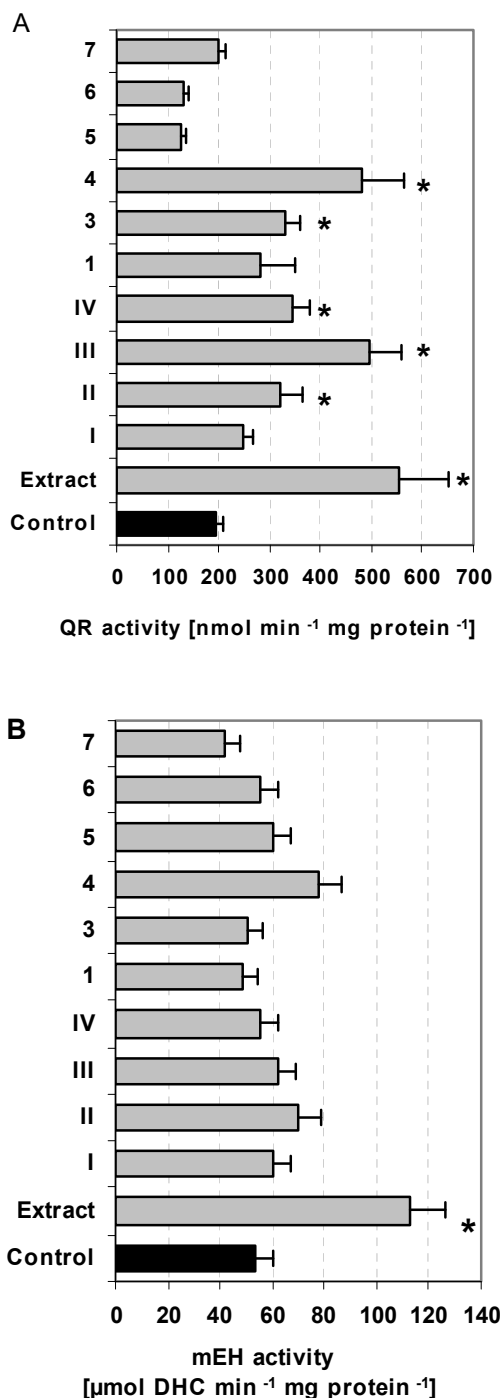


Fig. 4. The anti-initiating activity through modulation of the carcinogen metabolism: the effect of treatment with 10 $\mu\text{g/ml}$ of each sample for 48 h on (A) QR and (B) mEH activities in Hepa1c1c7 cells. Data is expressed as mean \pm SD.

cated that only the treatment with the ethanolic extract and fraction III resulted in a remarkable enhanced activity of mEH ($P < 0.05$), as shown in Fig. 4B.

In oxidative stress and inflammation, excessive production of ROS and nitrogen species results in DNA damage and contributes to tumour initiation and promotion, which might ultimately lead to carcinogenesis. Cell protection from external damage largely depends on the availability and activity of cellular antioxidants, which maintain homeostatic control of ROS (Sun *et al.*, 2004). An altered balance of ROS directly affects cellular proliferation, apoptosis, and senescence. Consequently, scavenging of the physiologically relevant ROS, including OH^\bullet and ROO^\bullet , represents an effective strategy in preventing tumour initiation and promotion.

Compounds **1** and **4** and fraction III were found to be the strongest ROO^\bullet scavengers as indicated by their high ORAC units compared to the trolox capacity and their high dose-dependent slope (Fig. 5B). Although **1** and fraction IV exhibited a relatively strong scavenging affinity against OH^\bullet , they showed low affinity against ROO^\bullet as indicated by their low ORAC- OH^\bullet values (<1 ORAC unit). The extract, fractions I and II, and **4** were found to be the strongest OH^\bullet scavenger as indicated by their high ORAC units compared to the trolox capacity and their high dose-dependent slope (Fig. 5A). Although the extract and fraction I exhibited a strong scavenging affinity against OH^\bullet , they showed low affinity against ROO^\bullet (ORAC- $\text{ROO}^\bullet < 1$). Generally, in Fig. 5 the samples that have ORAC values lower than 1 unit are not presented. Before the ORAC assay, we tested **1**, and found that it had no autofluorescence at the assay excitation and emission wavelength.

The comet assay allows the detection of diverse kinds of DNA alterations such as double-strand breaks, single-strand breaks, alkali-labile sites, incomplete repair sites, and cross-links (Tice *et al.*, 2000). Induction of DNA damage by the known inducer methyl methanesulfonate (MMS) led to a significant dramatic increase in the DNA damage ($P < 0.001$), as indicated by the length of the comet tail and the tail moment compared to their corresponding values in the untreated control cells, as shown in Table I. The human lymphocytes were treated with extracts 1 h before they were incubated with MMS for 3 h. The results revealed that the extract, fractions I and III, and **5** were strong

inhibitors of the MMS-induced DNA damage ($P < 0.01$), in contrast to fractions II and IV, which were moderate inhibitors ($P < 0.05$) as revealed from the inhibited tail moment (Table I).

Flavonoids and other polyphenolic plant constituents have been suggested to have preventive properties both at the initiation and the promotion stages of chemically induced carcinogenesis. At the cancer initiation stage, mainly in cell cul-

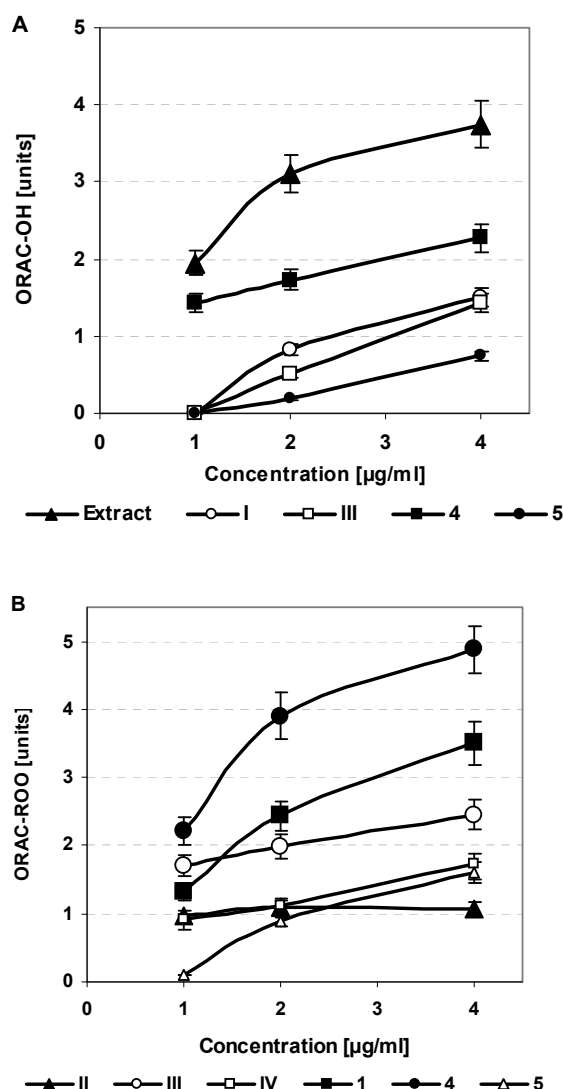


Fig. 5. The antioxidant capacity of different concentrations of the samples, as assayed by the ORAC assay, against (A) OH^\bullet and (B) ROO^\bullet radicals. Data represents only samples with ORAC unit >1 .

Table I. DNA damage measured by the comet assay. Human lymphocytes were treated with MMS, a known DNA damage inducer, and 10 $\mu\text{g/ml}$ of the extract and its constituents. The data is presented as mean \pm S.E. of the comet tail moment and length.

| Treatment | DNA damage ($n = 3$) | | <i>P</i> value for tail moment |
|----------------|------------------------|-------------------------------|--------------------------------|
| | Tail moment | Tail length [μm] | |
| Control | 0.48 \pm 0.41 | 26.26 \pm 2.71 | |
| MMS | 9.88 \pm 1.75 | 64.1 \pm 7.23 | < 0.01 ^a |
| MMS + Extract | 2.23 \pm 0.11 | 22.1 \pm 3.10 | < 0.01 ^b |
| MMS + I | 2.12 \pm 1.08 | 19.4 \pm 1.97 | < 0.01 ^b |
| MMS + II | 6.25 \pm 0.06 | 39.4 \pm 3.32 | < 0.05 ^b |
| MMS + III | 1.13 \pm 0.08 | 18.2 \pm 2.87 | < 0.01 ^b |
| MMS + IV | 6.23 \pm 0.08 | 18.2 \pm 2.87 | < 0.05 ^b |
| MMS + 1 | 7.35 \pm 0.27 | 22.4 \pm 1.98 | > 0.05 ^b |
| MMS + 3 | 8.11 \pm 0.38 | 61.29 \pm 9.22 | > 0.05 ^b |
| MMS + 4 | 9.55 \pm 0.09 | 33.04 \pm 2.26 | > 0.05 ^b |
| MMS + 5 | 2.37 \pm 0.02 | 38.44 \pm 3.91 | < 0.01 ^b |
| MMS + 6 | 9.40 \pm 0.036 | 63.41 \pm 8.98 | > 0.05 ^b |
| MMS + 7 | 8.41 \pm 0.045 | 34.63 \pm 4.11 | > 0.05 ^b |

^a Statistically significant increases compared to untreated control cells.

^b Statistically significant increases compared to MMS-treated cells.

ture studies, flavonoids have clearly been shown to affect many of the carcinogen bioactivating steps necessary for the covalent binding of the carcinogen to cellular DNA, including the major bioactivating CYP1A enzyme (Pervaiz, 2003).

Salvigenin (**3**), which is a trimethoxy derivative of apigenin, was found to enhance the thiol content and QR, while apigenin itself was reported to be less active. Our results are in agreement with the published data, which showed higher cancer chemopreventive properties of the methylated derivatives than free hydroxylated flavonoids have. Also, the methylation showed higher metabolic stability and membrane transport in the intestine/liver, thus improving the oral bioavailability (Thomas, 2007). On the other hand, derivatization of luteolin at position 7 (in **5** and **6**) largely diminished the activity in comparison with luteolin (**4**) as shown from the low CYP1A, QR and mEH activities. Glycosides at 3'-position were reported to decrease the QR activity of quercetin (Williamson *et al.*, 1996). Luteolin 7-*O*- β -arabinoside (**5**) induced GST and inhibited DNA damage more than the 7-*O*-glucoside derivative **6**, which suggests the rule of sugar substitution as an important factor in chemoprevention activity. The present study indicated that the ethanolic extract of *S. disermas*, its fraction III, and **4** were active tumour anti-initiating and multipotent blocking agents.

Taken together, the *S. disermas* extract and fraction III were promising radical scavengers, inhibitors of CYP1A activity, inducers of GST, QR, mEH and thiol content, and inhibitors of DNA damage. Fraction I possessed a radical scavenging activity to OH \cdot and inhibited DNA damage, while fraction II enhanced the QR activity and inhibited DNA damage. Additionally, fraction IV induced the GST and QR activities and inhibited DNA damage. On the other hand, **3** was an enhancer of thiol content and QR activity, while **4** was an inhibitor of CYP1A activity, an inducer of QR activity, and radical scavenger of ROO \cdot , and **5** was an inducer of GST activity and inhibitor of DNA damage.

Material and Methods

Materials

UV: Perkin Elmer Lambda 15 UV/Vis spectrophotometer. IR: Perkin Elmer 1600 Series FT-IR spectrometer. NMR: Varian Unity 500 (500 MHz). EIMS: Finnigan MAT 731 (70 eV). ESIMS: Finnigan LCQ. Solvent mixture: BAW (*n*-butanol/acetic acid/water 4:1:5 upper phase). Paper chromatography (PC): Whatman No. 3 (46 \times 57 cm). Leaves of *S. disermas* were collected in Pretoria, South Africa. A voucher specimen of *S. disermas* (Ahmd-4) was identified and deposited at the H.

G. W. J. Schweickerdt Herbarium (PRU 95054), University of Pretoria, South Africa.

Isolation of bioactive compounds

The leaves of *S. disermas* (550.0 g) were extracted with ethanol (2×2 l) for 48 h at room temperature ($\pm 25^\circ\text{C}$). The ethanolic extract was filtered and concentrated under vacuum. The total extract (10.0 g) was applied to a polyamide 6S column and eluted with H_2O followed by $\text{MeOH}/\text{H}_2\text{O}$ mixtures of decreasing polarities to yield four main fractions (I, II, III, IV). Fractions I and II were subjected separately to preparative paper chromatography (3 mm) using BAW and 15% acetic acid/water as eluents. The separated bands were scraped off with 70% methanol and purified by rechromatography on a Sephadex LH-20 column using methanol to get pure rosmarinic acid (**1**, 6.2 mg), caffeic acid (**2**, 4.5 mg), luteolin (**4**, 6.4 mg), luteolin 7-*O*- β -arabinoside (**5**, 12.5 mg), and luteolin 7-*O*- β -glucoside (**6**, 17.0 mg). Fraction III was applied to silica gel column chromatography (CC, 7×120 cm) eluted with *n*-hexane/ethyl acetate mixtures of increasing polarity (0 to 100%). Salvigenin (**3**, 26.0 mg) was purified from fraction III eluted with *n*-hexane/ethyl acetate (1:1) using a Sephadex LH-20 column eluted with methanol. Fraction II gave pure ocotillol II (**7**, 40.0 mg) using a silica gel column eluted with *n*-hexane/ethyl acetate (7:3). The isolated compounds were identified by recording their spectroscopic data (^1H and ^{13}C NMR, UV, MS) and comparing them with those in the literature.

Cell culture

Murine hepatoma cells (Hepa1c1c7) were purchased from the American Type Culture Collections. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM). Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulfate, and 250 ng/ml amphotericin B. Cells were maintained in humidified air containing 5% CO_2 at 37°C . The extract, fractions and compounds were dissolved in DMSO (99.9%) and diluted 1000-fold. In all the cellular experiments, results were compared with DMSO-treated cells. All cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark).

Evaluation of carcinogen metabolizing enzymes

The cytochrome P450 1A (CYP1A) activity was determined by the rate of dealkylation of 3-cyano-7-ethoxycoumarin (CEC) to the fluorescent 3-cyano-7-hydroxycoumarin according to Crespi *et al.* (1997) and modified by Gerhäuser *et al.* (2003). Homogenates from cultured Hepa1c1c7 cells induced with β -naphthoflavone were used as a source of CYP1A. A final concentration of $1.0 \mu\text{g}/\text{ml}$ of the extract, fractions and compounds was used.

Hepa1c1c7 cells (10^6) were incubated with the extract, fractions and compounds ($10.0 \mu\text{g}/\text{ml}$) for 48 h. GST activity was measured in the cell lysate according to Habig *et al.* (1974). GST were normalized to the protein content as measured by the bicinchoninic acid assay (Smith *et al.*, 1985). The QR activity was determined by measuring the reduction of 2,6-dichloroindophenol (Yu *et al.*, 2000). The specific QR activity was expressed as nmol of 2,6-dichloroindophenol reduced by 1 mg of protein within 1 min. The mEH enzyme activity was assessed by the production rate of 7-(29,39-dihydroxy)propoxycoumarin (DHC) from 7-glycidoxycoumarin (GOC), as described by Inoue *et al.* (1993). The enzyme activity was expressed as $\mu\text{mol DHC min}^{-1} \text{mg protein}^{-1}$. The total cellular thiol concentration was determined by the glutathione disulfide reductase/5,5'-dithiobis(2-nitrobenzoic acid) enzymatic cycling procedure (Griffith, 1981). The thiol content in the cell lysate was calculated in comparison with a GSH standard curve.

Oxygen radical absorbance capacity (ORAC)

The radical absorbance capacity of the extract, fractions and compounds was tested against two physiological radicals, peroxy (ROO^\bullet) and hydroxyl (OH^\bullet), by a kinetic ORAC assay (Cao and Prior, 1999) as modified by Gamal-Eldeen *et al.* (2004). One ORAC unit equals the net protection of α -PE produced by $1.0 \mu\text{M}$ 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox). In this assay, different concentrations (1.0, 2.0, and $4.0 \mu\text{g}/\text{ml}$) of the extract, fractions and compounds were used, and their antioxidant capacity against the physiological radicals ROO^\bullet and OH^\bullet was calculated.

Comet assay

Human lymphocytes were separated from peripheral blood, which was obtained from healthy male human volunteers, in a heparinized container from the Medical Unit, National Research Centre, Cairo, Egypt. Lymphocytes (10^6 cells) were incubated with $30\text{ }\mu\text{g/ml}$ MMS for 3 h in Iscove's medium, to induce DNA damage, in the presence and absence of test samples. Lymphocytes were treated with the extract, fractions and compounds ($10.0\text{ }\mu\text{g/ml}$) for 1 h before incubation with MMS for 3 h. The comet assay was performed according to McKelvey-Martin *et al.* (1997).

Statistical analysis

In the comet assay, the DNA damages were quantified as tail moment (the DNA product in the tail and the mean migration distance in the tail) and compared with untreated and MMS-treated cells by the nonparametric Mann-Whitney U test. Student's t test was used to analyze the normally distributed tail migration and head diameter. In the other tests data were analyzed by using one-way ANOVA followed by the Tukey *post hoc* test. $P < 0.05$ indicated statistical significance.

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