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Antioxidant activity and inhibition of aflatoxin B_1-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L.

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ABSTRACT

The effect of extracts obtained from *Rhamnus alaternus* L. leaves on genotoxicity and SOS response induced by aflatoxin B_1 (10 μg/assay) as well as nifuroxazide (20 μg/assay) was investigated in a bacterial assay system, i.e., the SOS chromotest with *Escherichia coli* PQ37. The evaluation of the mutagenic and antimutagenic actions of the same extracts against the sodium azide (1.5 μg/plate)-induced mutagenicity was assayed using the *Salmonella typhimurium* assay system. The *R. alaternus* tested extracts exhibited no genotoxicity either with or without the external S9 activation mixture. However, all the extracts, particularly aqueous extract (A) and its chloroformic fraction (A_2) significantly decreased the genotoxicity induced by aflatoxin B_1 and nifuroxazide. Moreover, the different extracts showed no mutagenicity when tested with *Salmonella typhimurium* strains TA1535 and TA1538 either with or without the S9 mix. Aqueous extract as well as its A_2 fraction exhibited the highest level of protection towards the direct mutagen, sodium azide-induced response in TA1535 strain with mutagenicity inhibition percentages of 83.6% and 91.4%, respectively, at a dose of 250 μg/plate. The results obtained by the Ames test assay confirm those of SOS chromotest. These same active extracts exhibited high xanthine oxidase (XOD) inhibiting with respective IC_{50} values of 208 and 137 μg/ml, and superoxide anion-scavenging effects (IC_{50} values of 132 and 117 μg/ml) when tested in the XOD enzymatic assay system. Our findings emphasize the potential of *R. alaternus* to prevent mutations and also its antioxidant effect.

1. Introduction

Exposure to genotoxic chemicals present in food, in the environment and used in medical treatment can alter the genetic material permanently and thus may lead to cancer [1]. At present, there are several antigenotoxicity assays available, which include the micronucleus test, somatic mutation and recombination test (SMART), sister chromatid exchange (SCE) assay and the single cell gel electrophoresis (SCGE) or comet assay. The above-mentioned assays may involve a longer analysis time, a high cost, and specialized skill or may require addition of expensive reagents. Therefore, short-term bacterial assays: Ames test and the SOS chromotest assay are useful and give an

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estimation of the genotoxic/antigenotoxic potential of substances [2].

Antigenotoxic plant extracts can counter or prevent the adverse effects caused by DNA damaging chemicals. In fact, researches, however, have shown that plant-derived polyphenolic compounds exert antigenotoxic property [3,4].

Accumulating data from *in vitro* and short-term *in vivo* studies as well as long-term carcinogenicity studies with chemically treated animals continue to show that phytochemicals could possess antimutagenic and anticarcinogenic effects [5]. Additionally, epidemiological studies support that chemopreventive effects are associated with the intake of plant materials [6]. However, under some experimental conditions, these phytochemicals exhibit genotoxic and/or mutagenic effects by themselves or potentiate the effect of other xenobiotics [7]. Thus it is of paramount importance to investigate the circumstances under which phytochemicals, used in traditional medicine exhibit beneficial and harmful effects [8].

On the other hand, oxidative stress, caused by reactive oxygen species (ROS), is known to cause the oxidation of biomolecules leading to cellular damage. The tissue injury caused by ROS may include DNA and protein damages, and oxidation of important enzymes. These events could consequently lead to the occurrence of various free radical-related diseases. In the human body, the toxic effects of ROS are combated regularly by a number of endogenous defence and protective mechanisms which include various enzymes and non-enzymatic antioxidants. These self-defence systems may also be supported by antioxidative compounds taken as foods, cosmetics and herb medicine particularly in the elderly [9].

Recently, natural foods and food derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage. Fruits, vegetables and herb medicines contain many antioxidant compounds, including carotenoids, thiols, vitamins such as ascorbic acid, tocopherols, flavonoids, and other phenolics [10].

The plant *R. alaternus* L. (Rhamnaceae), a wild species of the Mediterranean region, is commonly found growing in wilds around the farmlands in the North of Tunisia. It is known locally as "Oud el-Khir". The sheet of *R. alaternus* is laxative, purgative and has a strongly hypotensive activity [11]. The decoction of the air parts of this plant seems to be effective in the treatment of the hepatic complications while the bark in a meat bubble is employed in the North Africa countries against certain dermatological and hepatic affections. Other reported indications are the treatment of the burns and odontology and for the ocular care [12,13]. At present, scientific information reported on chemical and biological properties of *R. alaternus* remains limited. From the literatures, this plant species is known to contain compounds such as emodin, alaternin, quercetin, kaempferol, chrysophanol and physcion [14,15]. Although this plant species has been widely used as folk medicine in Tunisia and many other North African countries, it remains unknown if certain therapeutic claims of the *R. alaternus* preparations are derived from its antioxidant and/or antigenotoxic activities.

In this study, our aim was to examine the antimutagenic and antigenotoxic capacities of different *R. alaternus* extracts using SOS chromotest and Ames test assays. The objective here was also, to evaluate the antioxidant capacity of the *R. alaternus* extracts by the XOD system generated superoxide anion radical inhibition assay.

2. Materials and methods

2.1. Plant material

The leaves of *R. alaternus* were collected in the region of Tabarka situated in the western North of Tunisia in November 2006. Botanical identification was carried out by Dr. Ben Tiba (Institut Supérieur d'Agronomie, Chott-Mariam, Tunisia), according to the flora of Tunisia [16]. A voucher specimen (Ra-12-004) has been deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, Tunisia. The leaves were shade dried, powdered and stored in a tightly closed container for further use.

2.2. Preparation of plant extracts

The fresh leaves of *R. alaternus* L. were dried at room temperature and reduced to coarse powder. One hundred grams of the powdered leaves were extracted with boiling water (1 L) for 15–20 min. After filtration, the crude extract obtained was frozen and lyophilized, leading to the aqueous extract (A) which was dissolved in water. A_1, A_2 and A_3 were respectively the chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, obtained by a liquid–liquid separation. These fractions were concentrated to dryness and the residues were kept at 4 °C. Then, they were dissolved in dimethyl sulfoxide (DMSO).

Petroleum ether (PE) and Chloroform ($CHCl_3$) extracts were obtained by Soxhlet extraction (6 h) using 100 g of the powdered leaves and 1 L of solvent. These two extracts, with different polarities, were also concentrated to dryness and the residue was kept at 4 °C. They were dissolved in DMSO.

In the present study, six extracts were investigated. The doses of extracts we tested in both *Salmonella* microsome assay (10, 50, 250 and 350 μg/plate), SOS chromotest (10, 50 and 250 μg/plate) and xanthine/xanthine oxidase enzymatic assay (50, 150 and 300 μg/ml, respectively) are in accordance with our previous investigations [17–21], where a number of preliminary dose-finding tests involving a number of plant extracts were conducted. This means that the doses were suitable for testing the majority of the extracts, however, not necessarily all. Therefore, some extracts may be toxic at one or more of the applied doses.

2.3. Chemicals

O-Nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-nitrophenylphosphate (PNPP) were purchased from Merck (Dramstadt, Germany). The positive mutagens AFB_1, nifuroxazide and sodium azide (SA) were purchased from Sigma–Aldrich (St. Louis, MO, USA); histidine, biotine and agar–agar from Difco (Detroit, MI, USA); Aroclor 1254 from

Supelco (Bellafonte, PA, USA); the Oxoid Nutrient Broth No. 2 from Fluka Biochemika (Buchs, Switzerland); the xanthine (X) and the xanthine oxidase (XOD) from Sigma (St. Louis, MO, USA); the allopurinol from Sigma Chemical Co. (St. Louis, MO, USA) and the DMSO from Sigma–Aldrich (Seelze, Germany).

2.4. Bacterial tester strains

Salmonella typhimurium strains TA1538 and TA1535 which are histidine-requiring mutants, and *Escherichia coli* PQ37 strains were kindly provided by Pr. I. Felzen swalb (Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, RJ, Brazil) and maintained as described by Maron and Ames [22]. The genotypes of the Ames test strains were checked routinely for their histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvrB* mutation) and for the presence of the R factor. *S. typhimurium* TA1535 strain which contains the base-pair substitution mutation *hisG46* [23] is known to be more responsive to certain mutagens such as sodium azide [22] while *S. typhimurium* TA1538 strain is a frame-shift sensitive strain which contains the *hisD3052* mutation.

E. coli PQ37 has the genotype *F⁻ thr leu his*$^{-4}$ *pyr D thr galE galK lacU169 sRP300::Tn::10 rpoB rpsL uvrA rfa trp::Muc+ sfiA::Mud* (*Apy lac*) and the construction details of this strain were described by Quillardet and Hofnung [24].

Frozen permanent copies of the tester strain were prepared and stored at −80 °C.

2.5. Metabolic activation

The S9 microsome fraction is prepared from the livers of rats treated with Aroclor 1254 [22]. The activation mixture for the Ames test contained 8 mM magnesium chloride hexahydrate ($MgCl_2{\cdot}6\,H_2O$), 32.5 mM potassium chloride (KCl), 5 mM glucose-6-phosphate (G6P), 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 0.1 M sodium phosphate buffer, pH 7.4, and S9 fraction at a concentration of 492 μg protein/ml of mix.

For the SOS chromotest assay, the composition of S9 mix is the following: 1.65 M potassium chloride (KCl); 0.4 M magnesium chloride hexahydrate ($MgCl_2{\cdot}6\,H_2O$); 1 M glucose-6-phosphate (G6P), 0.1 M nicotinamide adenine dinucleotide phosphate (NADP), 0.4 M pH 7.4 Tris–buffer, Luria broth medium at a concentration of 0.61 ml/ml of mix; S9 fraction at a concentration of 1230 μg protein/ml of mix. The S9 fraction was stored at −80 °C.

2.6. Ames fluctuation assay

The Ames fluctuation assay is based on the most widely used and validated bacterial reverse-mutation test, known as the Ames test [25]. The test employs a mutant strain of *S. typhimurium*, carrying a mutation in the operon coding for histidine biosynthesis.

The TA1535 and TA1538 strains were used in the present study. When these bacteria are exposed to mutagenic agents, reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs. Tests were conducted under aseptic conditions.

2.6.1. Salmonella-microsome assay

The mutagenicity assay with *S. typhimurium* was performed as described by Maron and Ames [22]. The test is based on the plate incorporation method, using *S. typhimurium* test strains TA1535 and TA1538 with and without an exogenous metabolic system: S9 fraction in S9 mix. The test strains from frozen cultures were grown overnight for 12–14 h at 37 °C in the Oxoid Nutrient Broth No. 2. Various concentrations of each extract were added to 2 ml of top agar, supplemented with 0.5 mM L- histidine and 0.5 mM D-biotine, mixed with 100 μl of bacterial culture (approximate cell density 2×10^8–5×10^8 cells/ml) and then poured onto a plate containing minimum agar. The plates were incubated at 37 °C for 48 h and his$^+$ revertant colonies were counted. The influence of metabolic activation was tested by adding 500 μl of S9 mixture. Negative and positive control cultures gave number of revertants per plate that were within the normal limits found in the laboratory.

Results are expressed as mutagenic index (MI) calculated as follows:

$$\mathrm{MI} = \frac{\text{number of his}^+ \text{ revertants induced in the sample}}{\text{number of spontaneous his}^+ \text{ revertants in the negative control}}.$$

Data were collected with a mean ± standard deviation of three plates ($n = 3$).

2.6.2. Antimutagenicity testing

A modified plate incorporation procedure [26] was employed to determine the effect of extracts on sodium azide-induced mutagenicity. In brief, 0.5 ml of phosphate buffer was distributed in sterilized capped tubes in an ice bath, then 0.1 ml of test extracts and 0.1 ml of bacterial culture (prepared as described in mutagenicity test) were added. After vortexing gently and preincubating at 37 °C for 30 min, 2 ml of top agar supplemented with 0.05 M L-histidine and D-biotine were added to each tube and vortexed for 3 s. The resulting entire was overlaid on the minimal agar plate. The plates were incubated at 37 °C for 48 h and the revertant bacterial colonies on each plate were counted. The inhibition of mutagenicity (IM%) was calculated relative to those in the control group with the mutagen by the following formula:

$$\mathrm{IM}(\%) = \left[1 - \frac{\text{number of revertants on test plates} - \mathrm{SR}}{\text{number of revertants on control plates} - \mathrm{SR}}\right] \times 100.$$

where SR: spontaneous revertants

Each dose was tested in triplicate.

2.7. SOS chromotest assay

The SOS chromotest employs the error-prone DNA repair pathway of *E. coli* PQ37, also known as the SOS response, a complex regulatory network that is induced by

DNA-damaging substances [27]. The test involves incubation of the bacteria with the sample under investigation and subsequent determination of β-galactosidase (β-gal) activity (i.e. the level of SOS induction). Alkaline phosphatase (Ap) activity is also measured, as a toxicity control.

2.7.1. Antigenotoxic assay

The SOS chromotest was employed to determine the effect of the *R. alaternus* extracts on aflatoxin B_1 (AFB_1: indirect acting mutagen) and nifuroxazide (direct acting mutagen) induced genotoxicity. Genotoxicity and anti-genotoxicity assays were performed according to the procedure outlined by Quillardet and Hofnung [24]. Exponential-phase culture of *E. coli* PQ37 was grown at 37 °C in Luria Broth medium (1% bactotryptone, 0.5% yeast extract and 1% NaCl) plus 20 μg/ml ampicillin and diluted 1:9 into fresh medium; 100 μl aliquots were distributed into glass test tubes containing up various doses of *R. alaternus* extracts in a 0.6 ml final volume. The extracts were dissolved in DMSO or distilled water and tested in triplicate, with or without exogenous metabolic activation.

A positive control was prepared by exposure of the bacteria to either nifuroxazide or AFB_1. After 2 h of incubation at 37 °C, with shaking, 300 μl samples were used for assay of β-galactosidase (β-gal) and alkaline phosphatase (Ap) activities. In this assay, the β-galactosidase synthesis (*lacZ* gene) is dependent on *sfiA* activation and is used as a measure of SOS repair system induction. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. These enzyme activities were assayed in a Helios Alpha, Spectronic Unicam (Cambridge, England) spectrophotometer.

The SOS induction factor (IF) was calculated as the ratio of R_c/R_0, where R_0 and R_c are equal to (β-gal) activity/alkaline phosphatase (Ap) activity determined respectively in the absence and in the presence of the test compound at a concentration *c*. The IF in treated cells was obtained by comparing β-galactosidase and alkaline phosphatase activity in treated and untreated cells. The result was considered positive when the IF for β-galactosidase activity was >2.

For evaluation of the effect of *R. alaternus* extracts on induction of the SOS response by nifuroxazide (in the absence of the S9 activation mixture) and AFB_1 (in the presence of the S9 activation mixture), 20 μl of nifuroxazide solution (20 μg/assay) or AFB_1 solution (10 μg/assay) were added into tubes with 20 μl of tested concentration of extracts. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity induced by either nifuroxazide or AFB_1 according to the formula:

$$\text{Inhibition}\,(\%) = \left[100 - \frac{(\text{IF}_1 - \text{IF}_0)}{(\text{IF}_2 - \text{IF}_0)} \times 100\right]$$

Where IF_1 is the induction factor in the presence of both test compound and mutagen, IF_2 the induction factor in the absence of the test compound and in the presence of mutagen and IF_0 the induction factor of the untreated cells.

2.8. Evaluation of XOD inhibition effect and superoxide radical scavenging activity

Both the inhibition of XOD activity and the superoxide anion scavenging activity were assessed *in vitro* in one assay. The inhibition of XOD activity was measured according to the increase in absorbance at 290 nm as proposed by Cimanga et al. [28]; while the superoxide anion scavenging activity was detected spectrophotometrically by the nitrite method described by Oyangagui [29], with some modifications introduced by Russo et al. [30].

Briefly, the assay mixture consisted of 100 μl of the test extract (50, 150 and 300 μg/ml), 200 μl xanthine (X) (7.6×10^3 μg/ml) as the substrate, hydroxylamine (6.6×10^3 μg/ml), 200 μl EDTA (3.7×10^3 μg/ml) and 300 μl distilled water. The reaction was initiated by adding 200 μl XOD (11 mU/ml) dissolved in phosphate buffer (KH_2PO_4, 2.8×10^6 μg/ml, pH 7.5). The assay mixture was incubated at 37 °C for 30 min. Before measurement of the uric acid production at 290 nm, the reaction was stopped by adding 0.1 ml of HCl (0.5 M). The absorbance was measured spectrophotometrically against a blank solution prepared as described above but replacing XOD with buffer solution. Another control solution, without the tested extract, was prepared in the same manner as the assay mixture to measure the total uric acid production (100%). The latter was calculated from the differential absorbance.

To detect the superoxide scavenging activity, 2 ml of the colouring reagent consisting of sulphanilic acid solution (300 μg/ml), *N*-(1-naphtyl) ethylenediamine dihydrochloride (5 μg/ml) and acetic acid (16.7%, v/v) were added. This mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 550 nm on a Helios Alpha, Spectronic Unicam (Cambridge, England) spectrophotometer.

For both inhibition of XOD activity and superoxide anion scavenging effect, allopurinol was used as a positive control.

The dose–effect curve for each tested extract was linearized by regression analysis and used to derive the IC_{50} values. Values are presented as mean ± standard deviation of three determinations.

2.9. Statistical analysis

Data were collected and expressed as the mean ± standard deviation of three independent experiments and analyzed for statistical significance from control, using the Dunnett test SPSS 11.5 Statistics Software (SPSS, Chicago, IL, USA). The criterion for significance was set at $p < 0.05$. IC_{50} values, from the *in vitro* data, were calculated by regression analysis.

3. Results

3.1. Mutagenic/genotoxic activity of R. alaternus extracts

In a series of experiments preceding the antimutagenicity studies, it was ascertained that the different amounts of extracts added to both the Ames and SOS chromtest indicator bacteria do not influence their viability.

Table 1
Mutagenic index of the *R. alaternus* extracts tested by the *S. typhimurium* TA1535 and TA1538 (his$^-$ reverse mutation) assay systems, without and with S9 activation

Extract	Concentration (μg/plate)	Mutagenic index (MI)			
		TA1535		TA1538	
		−S9	+S9	−S9	+S9
SR	0	14 ± 2 (1.00)	18 ± 1 (1.00)	21 ± 2 (1.00)	23 ± 2 (1.00)
PE	10	12 ± 2 (0.85)	16 ± 1 (0.88)	23 ± 3 (1.09)	27 ± 6 (1.17)
	50	17 ± 3 (1.21)	22 ± 1 (1.22)	28 ± 4 (1.33)	30 ± 8 (1.30)
	250	21 ± 2 (1.50)	26 ± 1 (1.44)	29 ± 8 (1.38)	31 ± 4 (1.34)
$CHCl_3$	10	15 ± 4 (1.07)	17 ± 1 (0.94)	20 ± 7 (0.95)	26 ± 5 (1.13)
	50	16 ± 3 (1.14)	19 ± 1 (1.05)	25 ± 5 (1.19)	25 ± 3 (1.08)
	250	20 ± 2 (1.42)	24 ± 1 (1.33)	31 ± 2 (1.47)	29 ± 3 (1.26)
A	10	13 ± 4 (0.92)	21 ± 1 (1.16)	22 ± 7 (1.04)	23 ± 5 (1.00)
	50	18 ± 3 (1.28)	20 ± 1 (1.11)	23 ± 5 (1.09)	28 ± 2 (1.21)
	250	24 ± 2 (1.71)	27 ± 1 (1.50)	27 ± 2 (1.28)	33 ± 3 (1.43)
A_1	10	16 ± 4 (1.14)	15 ± 1 (0.83)	24 ± 7 (1.14)	22 ± 5 (0.95)
	50	19 ± 3 (1.35)	18 ± 1 (1.00)	28 ± 5 (1.33)	31 ± 2 (1.34)
	250	22 ± 2 (1.57)	23 ± 1 (1.27)	32 ± 2 (1.52)	36 ± 3 (1.56)
	350	25 ± 1 (1.78)	30 ± 2 (1.66)	38 ± 2 (1.80)	44 ± 3 (1.91)
A_2	10	11 ± 4 (0.78)	19 ± 1 (1.05)	19 ± 3 (0.90)	24 ± 5 (1.04)
	50	14 ± 3 (1.00)	22 ± 1 (1.22)	22 ± 5 (1.04)	27 ± 2 (1.17)
	250	19 ± 2 (1.35)	28 ± 1 (1.55)	26 ± 2 (1.23)	34 ± 3 (1.47)
A_3	10	17 ± 4 (1.21)	16 ± 1 (0.88)	21 ± 7 (1.00)	25 ± 1 (1.08)
	50	16 ± 3 (1.14)	17 ± 1 (0.94)	25 ± 5 (1.19)	31 ± 4 (1.34)
	250	23 ± 2 (1.64)	20 ± 1 (1.11)	30 ± 2 (1.42)	35 ± 3 (1.52)

SR: spontaneous revertants; the numbers represent histidine revertants in terms of mean ± standard deviation ($n = 3$). Values in parentheses indicate the mutagenic index (MI). PE: petroleum ether extract; $CHCl_3$: chloroformic extract; A: aqueous extract; A_1, A_2 and A_3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.

In the Ames test, it was shown that in the presence of the different concentrations of *R. alaternus* extracts, the mutation frequencies didn't change significantly when compared to spontaneous ones. Indeed, none of the tested extracts induced significant increase of the revertants number neither in TA1535 nor in TA1538 strains, as well as with or without the S9 metabolic system (MI < 3). The results of Ames test with and without metabolic activation are reported in Table 1. In addition, it was revealed that tested extracts in the concentrations used have a very low effect on IF in the SOS chromotest with or without metabolic activation (Tables 2 and 3), except A_1 extract (IF = 1.54) which was marginally genotoxic at 250 μg/assay with S9 metabolic system.

3.2. Antimutagenicity assay

Dose of 1.5 μg/plate of sodium azide was chosen for the antimutagenicity study, since this dose was not toxic and induced 362 ± 8 revertants in *S. thyphimurium* TA1535. The inhibitory effects of different extracts from *R. alaternus* on the mutagenicity of positive mutagen using the plate incorporation assay are reported in Table 4. With *S. thyphimurium* TA1535 strain, the addition of aqueous extract (A) reduced sodium azide-induced mutagenicity and a clear dose–response effect was noticed. Indeed, at the concentrations of 10, 50 and 250 μg/plate, the A extract reduced the mutagenicity by respectively 34.8%, 64.1% and 83.6%. Whereas, the A_2 fraction was highly effective in reducing the mutagenicity caused by this direct mutagen

Table 2
Genotoxicity induced by *R. alaternus* extracts in the presence of the exogenous metabolic activation mixture (S9)

Extract	Dose (μg/assay)	ß-gal (U)	Ap (U)	IF
PC	10	13.42 ± 0.001	12.08 ± 0.003	5.29
NC	0	1.97 ± 0.002	9.32 ± 0.001	1.00
PE	10	3.21 ± 0.002	11.58 ± 0.001	1.32*
	50	3.59 ± 0.004	12.52 ± 0.002	1.36*
	250	3.54 ± 0.001	12.05 ± 0.001	1.39*
$CHCl_3$	10	3.25 ± 0.003	13.94 ± 0.002	1.11
	50	3.81 ± 0.002	13.64 ± 0.004	1.33*
	250	3.65 ± 0.001	12.23 ± 0.003	1.42*
A	10	3.25 ± 0.004	13.76 ± 0.002	1.12
	50	3.34 ± 0.001	13.29 ± 0.003	1.19
	250	3.58 ± 0.003	12.81 ± 0.003	1.33*
A_1	10	3.46 ± 0.001	13.52 ± 0.004	1.21
	50	3.56 ± 0.003	11.82 ± 0.001	1.43*
	250	3.62 ± 0.003	11.17 ± 0.003	1.54*
A_2	10	3.68 ± 0.001	13.70 ± 0.002	1.27*
	50	3.59 ± 0.001	12.82 ± 0.001	1.34*
	250	3.75 ± 0.003	11.94 ± 0.003	1.49*
A_3	10	3.81 ± 0.001	13.88 ± 0.002	1.30*
	50	4.03 ± 0.001	13.52 ± 0.001	1.41*
	250	4.09 ± 0.003	13.58 ± 0.003	1.43*

PC: positive control (AFB_1); NC: negative control; U: enzymatic unit; ß-gal: ß-galactosidase; Ap: alkaline phosphatase; IF: Induction factor; PE: petroleum ether extract; $CHCl_3$: chloroformic extract; A: aqueous extract; A_1, A_2 and A_3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.

* Significant difference between the control and treated groups, $p < 0.05$, $n = 3$ in each group.

Table 3
Genotoxicity induced by *R. alaternus* extracts in the absence of the exogenous metabolic activation mixture (S9)

Extract	Dose (μg/assay)	ß-gal (U)	Ap (U)	IF
PC	20	20.05 ± 0.003	13.27 ± 0.001	5.92
NC	0	2.77 ± 0.001	10.68 ± 0.002	1.00
PE	10	5.33 ± 0.001	15.40 ± 0.003	1.33*
	50	5.33 ± 0.002	14.20 ± 0.004	1.45*
	250	5.54 ± 0.002	14.26 ± 0.001	1.49*
$CHCl_3$	10	4.54 ± 0.000	12.66 ± 0.004	1.37*
	50	4.41 ± 0.003	14.40 ± 0.001	1.17
	250	4.33 ± 0.002	14.80 ± 0.000	1.12
A	10	4.29 ± 0.004	14.60 ± 0.002	1.13
	50	4.87 ± 0.001	13.93 ± 0.001	1.34*
	250	4.91 ± 0.002	13.46 ± 0.003	1.40*
A_1	10	4.83 ± 0.004	14.93 ± 0.002	1.24*
	50	5.20 ± 0.002	15.26 ± 0.002	1.31*
	250	5.37 ± 0.003	14.53 ± 0.002	1.42*
A_2	10	5.45 ± 0.000	15.53 ± 0.001	1.35*
	50	5.62 ± 0.002	15.40 ± 0.001	1.40*
	250	5.00 ± 0.004	13.33 ± 0.002	1.44*
A_3	10	4.54 ± 0.001	13.80 ± 0.002	1.26*
	50	4.79 ± 0.002	14.13 ± 0.003	1.30*
	250	5.91 ± 0.003	15.46 ± 0.001	1.47*

PC: positive control (nifuroxazide); NC: negative control; U: enzymatic unit; ß-gal: ß-galactosidase; Ap: alkaline phosphatase; IF: Induction factor; PE: petroleum ether extract; $CHCl_3$: chloroformic extract; A: aqueous extract; A_1, A_2 and A_3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.

* Significant difference between the control and treated groups, $p < 0.05$, $n = 3$ in each group.

Table 4
Inhibition of the mutagenicity (IM%) of Sodium azide (1.5 μg/plate) induced mutagenicity, by the *R. alaternus* extracts without metabolization, in *S. thyphimurium* TA1535 assay system

Extract	Concentration (μg/plate)	TA1535	
		NR ± S.D.	%IM
Spontaneous	0	14 ± 2	
Sodium azide	1.5	362 ± 8	0
PE	10	310 ± 5	14.9
	50	245 ± 7	33.6*
	250	196 ± 3	47.7*
$CHCl_3$	10	256 ± 4	30.5*
	50	183 ± 4	51.4*
	250	172 ± 8	54.6*
A	10	241 ± 7	34.8*
	50	139 ± 6	64.1*
	250	71 ± 4	83.6*
A_1	10	294 ± 5	19.5
	50	264 ± 6	28.2*
	250	199 ± 7	46.8*
	350	159 ± 6	58.3*
A_2	10	203 ± 4	45.7*
	50	95 ± 5	76.7*
	250	44 ± 3	91.4*
A_3	10	288 ± 6	21.3
	50	230 ± 4	37.9*
	250	152 ± 4	60.3*

NR: number of revertants; PE: petroleum ether extract; $CHCl_3$: chloroformic extract; A: aqueous extract; A_1, A_2 and A_3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.

* Significant difference between the control and treated groups, $p < 0.05$, $n = 3$ in each group.

with 45.7%, 76.7% and 91.4% at 10, 50 and 250 μg/plate, respectively.

The A_3 fraction showed a dose–response moderate antimutagenic activity as compared with A extract and its A_2 fraction with mutagenicity inhibitions of 21.3%, 37.9% and 60.3% at 10, 50 and 250 μg/plate, respectively. The highest used dose in this study (350 μg/plate of A_1 fraction) inhibited the sodium azide-induced mutagenicity by only 58.3%.

Using the same strain assay system, antimutagenic effect of PE and $CHCl_3$ extracts against sodium azide were relatively weak as compared with the aqueous extract and its A_2 and A_3 fractions. In fact, PE extract reduced the sodium azide-induced mutagenicity by 14.9%, 33.6% and 47.7% at 10, 50 and 250 μg/plate, respectively, while the $CHCl_3$ extract exhibited the mutagen reduction rates of 30.5%, 51.4% and 54.6% at the same doses.

3.3. Antigenotoxic assay

Doses of 20 μg/assay of nifuroxazide (directly acting mutagen), and 10 μg/assay of AFB_1 (indirectly acting mutagen) were chosen for the antigenotoxicity studies, since these doses were not toxic, induced a significant SOS response and they were the doses that give the maximum of mutagenicity for both nifuroxazide and AFB_1.

The inhibitory effects of the tested extracts on the mutagenicity induced by AFB_1 using the SOS chromotest are illustrated by Fig. 1. Increased concentrations of A extract as well as its A_1, A_2, and A_3 fractions and the $CHCl_3$ extract decreased AFB_1-induced genotoxicity. Indeed, the highest inhibition percentages of genotoxicity obtained with the above-mentioned extracts were respectively 72.9% (at a concentration of 10 μg/assay), 60.3% (at 250 μg/assay), 80.9% (at 250 μg/assay), 53.8% at the same concentration of 250 μg/assay and 71.6% (at 250 μg/assay). Whereas, PE extract showed a relatively weak efficiency in reducing AFB_1-induced genotoxicity, the IF of this mutagen was decreased respectively about 43% at 250 μg/assay. Except the aqueous extract which reduced AFB_1 genotoxicity with an inversed dose–response effect, the five remaining

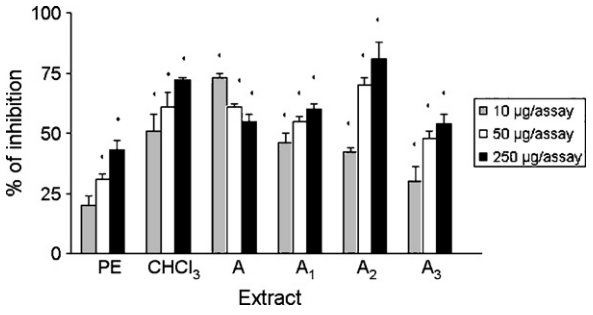

Fig. 1. Inhibition of AFB_1 (10 μg/assay)-induced mutagenicity in *E. coli* PQ37 assay system, in the presence of metabolic activation (S9), using the SOS chromotest. Symbols represent statistical significance from control (*$p < 0.05$).

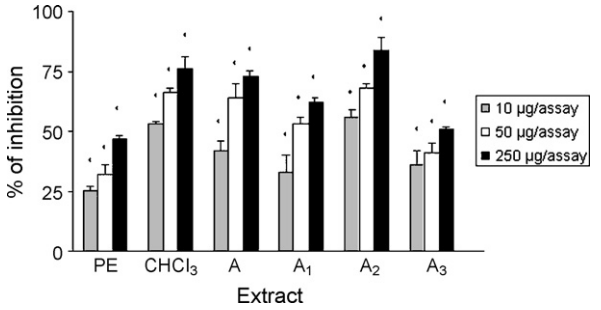

Fig. 2. Inhibition of nifuroxazide (20 μg/assay)-induced mutagenicity in *E. coli* assay system, using the SOS chromotest. Symbols represent statistical significance from control ($^{*}p < 0.05$).

extracts induced a decrease of the mutagen genotoxicity as a function of extract concentration.

Using nifuroxazide as mutagen, the induction of SOS response was also affected by the six tested extracts. As shown in Fig. 2, at 250 μg/assay, the IF of nifuroxazide decreased by 73.1%, 62.3%, 84.1%, 51.2%, 76.2% and 46.6% in the presence of A, A_1, A_2, A_3, $CHCl_3$ and PE extracts, respectively. This study showed that, towards nifuroxazide-induced mutagenicity, A_2 fraction was the most effective antimutagen while PE extract was the weakest one. In fact, in the presence at 10 μg/assay of A_2 and PE extracts, the IF of nifuroxazide decreased by, respectively, 55.9% and 25%. We can also notice that all the tested *R. alaternus* extracts induced a decrease of nifuroxazide genotoxicity in a dose–response manner.

3.4. Evaluation of XOD activity

The six *R. alaternus* extracts, and particularly the A_2 fraction and the A extract exhibited an inhibitory effect on XOD activity in a concentration dependent manner (Table 5). Indeed, at concentrations of 50, 150 and 300 μg/ml, the XOD inhibitory activities were 38.8%, 52.6% and 74.7% for A_2 and 30.2%, 43.4% and 61.9% for the A extract. The weakest enzyme inhibitory activity was shown in the presence of PE extract with a maximal inhibition percentage of 38% at 300 μg/ml. The IC_{50} values of the tested extracts were 271, 208 and 137 μg/ml for the $CHCl_3$, A and the A_2 fraction extracts, respectively. Whereas the PE, A_1 and A_3 fraction extracts have IC_{50} values superior than 300 μg/ml. In addition, data in Table 5 revealed that superoxide-scavenging abilities increased with concentration of the *R. alaternus* extracts. In fact, when compared to the other extracts, activity was strongest with the A_2 fraction at the three concentrations tested (31.3%, 62.3% and 84.2% inhibition at 50, 150 and 300 μg/ml, respectively). The studied extracts can be classified into four groups with regard to their scavenging activities: high activity, >75% inhibition (A extract and its A_2 fraction); moderate activity, 50– 74% inhibition (A_3 fraction and $CHCl_3$ extract) and low activity, 25–49% inhibition (A_1 fraction and PE extract).

In this assay, the IC_{50} values of the tested extracts were 210, 132, 117 and 278 μg/ml for the $CHCl_3$, A, A_2 and the A_3 fraction extracts, respectively. Whereas the PE and the A_1 fraction extracts have IC_{50} values superior than 300 μg/ml.

Based on data obtained by both assays, the order of efficacies of the extracts was $A_2 > A > CHCl_3 > A_3 > A_1 > PE$, but the activities were higher for the scavenging effect on superoxide anions than on the production of uric acid suggesting that *R. alaternus* extracts are better superoxide scavengers than XOD inhibitors.

On the other hand, we have previously shown that the aqueous extracts (A) and the A_2 fraction contained: 22.7% and 20.5% of phenolic compounds (gallic acid), respectively and 21.3% and 18.8% of flavonoids (quercetin), respectively; however, the PE, the $CHCl_3$ extracts, the A_1 and A_3 fractions contained 0.6%, 8%, 7.2% and 5.7% of phenolic compounds and 7.9%, 15.4%, 8.2% and 4.8% of flavonoids, respectively [31].

4. Discussion

XOD, a flavoprotein which catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid [32] is one of the main enzymatic sources of reactive oxygen species (ROS) *in vivo*. Under normal physiological conditions, the endogenous superoxide scavengers in the system protect tissues by neutralizing these radicals. This *in vivo* reaction is simulated in the *in vitro* model so as to use it as an analytical tool to evaluate the ROS scavenging abilities of natural products [33].

Results of the XOD inhibition assay showed that the uric acid inhibition as well as the superoxide scavenging activity of *R. alaternus* extracts might be caused by direct quenching of the radicals rather than a blockage of enzyme action of XOD. In fact, *R. alaternus* extracts are better radical scavengers than XOD inhibitors. In a

Table 5
inhibition of XOD and scavenging of superoxide anions ($O_2^{\bullet -}$) by *R. alaternus* extracts at the indicated concentrations[a]

Extract/dose (μg/ml)	(XOD) inhibition (%)			($O_2^{\bullet -}$) scavenging (%)		
	50	150	300	50	150	300
PE	5.2 ± 0.5	24.1 ± 3	38.0 ± 3*	12.1 ± 2	37.1 ± 4*	44.2 ± 5*
$CHCl_3$	9.9 ± 1	32.8 ± 4*	55.4 ± 6*	20.2 ± 2	42.2 ± 6*	63.3 ± 4*
A	30.2 ± 5*	43.4 ± 2*	61.9 ± 7*	25.1 ± 3	57.5 ± 7*	76.0 ± 8*
A_1	12.5 ± 2	22.3 ± 3	37.7 ± 2*	16.6 ± 3	29.1 ± 5*	47.8 ± 6*
A_2	38.8 ± 6*	52.6 ± 3*	74.7 ± 4*	31.3 ± 1*	62.3 ± 2*	84.2 ± 4*
A_3	19.5 ± 3	33.6 ± 1*	47.3 ± 5*	26.5 ± 2	40.2 ± 3*	51.3 ± 2*

PE: petroleum ether extract; $CHCl_3$: chloroformic extract; A: aqueous extract; A_1, A_2 and A_3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.

[a] Values expressed as means ± SD ($n = 3$).

* Significant difference between the control and treated groups, $p < 0.05$.

previous study, we have shown that the tested extracts contained total phenolic compounds (as gallic acid equivalents) in the following order: A > A_2 > $CHCl_3$ > A_1 > A_3 > PE and total flavonoids (as quercetin equivalents) in the following order: A > A_2 > $CHCl_3$ > A_1 > PE > A_3 [31]. In our study, the antioxidant order of the tested extracts follows almost their total polyphenol and total flavonoid content ones, and the correlation observed between the activity and the concentrations used suggests the involvement of active constituents in the extracts that can scavenge superoxide anions and inhibit XOD activity. In fact, polyphenols are considered to be the most active antioxidant derivatives in plants [34]. However, it has been shown in other studies [35] that the phenolic content does not necessarily correspond to the antioxidant activity. The latter is the result of the combined activity of a wide range of compounds, including phenolics, peptides, organic acids and other components [36].

As far as antioxidants has attracted much interest with respect to their protective effect against free radical damage that may be the cause of many diseases including cancer, antigenotoxic activity of *R. alaternus* extracts was invested in the present study. The SOS chromotest was developed as an alternative to the Ames test and has been recommended for routine use in environmental applications requiring the assessment of genotoxic activity [37]. These two assays are widely used particularly for testing genotoxic and anti-genotoxic potentials of extracts from medicinal plants [17–21,38-41].

In our study, none of the tested extracts exhibit mutagenic/genotoxic effect neither in the Ames test nor with the SOS chromotest assay, as well in the presence or the absence of enzymatic metabolization. Indeed, in the Ames test, an extract was considered mutagenic if the number of revertants per plate was at least tripled over the spontaneous revertant frequency in both TA1535 and TA1538 strains [22,42] while, in the SOS chromotest, a genotoxic extract must have an IF > 2 [42]. This suggests that DNA does not seem to be a relevant target for *R. alaternus* tested extracts and they did not produce DNA lesions that block DNA synthesis, leading to the induction of SOS system. The absence of genotoxicity is not a characteristic of all natural products in use, since other medicinal plants, assayed with the SOS chromotest and the Ames test, in the presence or not of the S9 mix, have resulted positive for genotoxicity [38]. This result added to others such as the antioxidant effect and the high total flavonoid and polyphenol contents in the active *R. alaternus* extracts may corroborated the traditional use of this plant species in Tunisia and other North African countries [12,13].

On the other hand, anti-genotoxic properties elicited by plant species have a full range of prospective applications in human healthcare. Herbal remedies and phytotherapy drugs containing active principles are currently developed to protect against electrophile (e.g., free radicals) attack to DNA and its widespread outcomes such as ageing and cancer.

The antignotoxic study of the *R. alaternus* extracts evaluated by the SOS chromotest towards the indirect mutagen AFB_1 and the direct mutagen nifuroxazide, revealed significant antigenotoxic effects of these extracts, particularly A, A_2, $CHCl_3$ and A_1 extracts. These results indicate that these active extracts are able to interact and neutralize electrophiles such as nifuroxazide or may inhibit microsomal activation of AFB_1 to electrophilic metabolites. *R. alaternus* extracts may act, as described for others polyphenols such as flavonoids, by inhibiting the mutation or initiation caused by inhibition of pro-mutagen activation and trap the electrophiles by chemical reaction or conjugation; also to exert their antioxidant activity or scavenging of reactive oxygen species [43]. In this study, we suspect an eventual correlation between antioxidant and antigenotoxic effects of *R. alaternus* extracts, as suggested for other molecules and/or plant extracts by numerous authors [44,45]. Antioxidant activity expressed by the *R. alaternus* extracts may provide a common mechanism for inhibiting the genotoxicity of both AFB_1 and nifuroxazide. However, the inhibition of mutagenesis is often complex and acts through multiple mechanisms.

In the SOS chromotest, extracts from *R. alaternus* inhibited strongly nifuroxazide-induced mutagenicity compared to the results obtained against AFB_1-induced mutagenicity, suggesting that these extracts did not necessitate metabolic activation. Anti-genotoxic activity of the tested extracts may be ascribed to flavonoids [46], tannins [47] and total polyphenols [48]. These metabolites were previously detected in *R. alaternus* extracts [31] in the same order as their antigenotoxic effect. We cannot, however, exclude the possibility that other compounds with antigenotoxic properties participate in the inhibitory effect of mutagens.

In this study, the results obtained by the Ames test assay confirm those of SOS chromotest. Also, our results confirm previous findings such as DPPH· free radical scavenging activity of these extracts and their antimutagenic effect using other Ames bacterial strains TA98 and TA100 [20].

Towards AFB_1, the weak antimutagenic activity of the aqueous extract when large excess was added to the assay system could be explained by the inhibition of the penetration through the cell membrane at high doses of molecules which are implied in the mutagenic inhibitory effect. Besides, the highest antioxidant and antigenotoxic activities were observed in the A_2 fraction compared to the aqueous extract A. This result could be explained by the presence of active compounds diluted and/or masked by various components in the whole crude aqueous extract and which are more accessible in the A_2 fraction.

5. Conclusion

The present study has demonstrated that some *R. alaternus* extracts possess potent antioxidant and antigenotoxic activities, of which could be derived from compounds such as flavonoids and phenols. The antigenotoxic activity could be ascribed, at least in part, to their antioxidant properties but we cannot exclude other additionally mechanisms. The results obtained by the Ames test assay confirm those of SOS chromotest. These antioxidant and antigenotoxic activities could have contributed, at least partly, to the therapeutic benefits of the certain traditional claims. The results presented here could be an additional argument to support the use of this species in the North African traditional

medicine. Furthermore, *R. alaternus* extracts could give rise to anticarcinogenic agents and could be promising candidates for further studies designed to obtain more evidence on their components with potential chemo-preventive activity.

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