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In vitro Toxicity and Genotoxic Activity of Aqueous Leaf Extracts From Four Varieties of *Olea europea* (L)

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Abstract

Aim:

Despite its therapeutic value almost nothing is known about potential adverse health effects of *Olea europea L*. We therefore investigated the *in vitro* toxicity and genotoxicity of leaf extracts of this plant.

Material and Methods:

Extracts from olive tree leaves were obtained from four different regions in Tunisia. We investigated the *in vitro* toxicity, genotoxicity and antigenotoxicity of their aqueous extracts using the neutral red (NR) uptake, Vitotox and alkaline comet assays.

Results:

None of the extracts were found to be toxic and none of them were genotoxic, although some doubt exists for the extract obtained at Meski (North of Tunisia). On the basis of the Vitotox test only, none of the extracts appeared to have antigenotoxic (or cogenotoxic) properties.

Discussion:

The negative genotoxicity underline the safe use of the leaves, for example, as hypoglycemic and antidiabetic preparations. Lack of antigenotoxicity may indicate that the previously reported anticancer effects do not result from protection against genotoxicity.

HIGHLIGHTS

- We investigated the *in vitro* toxicity and genotoxicity of aqueous extracts of olives
- The neutral Red Uptake test, Vitotox and alkaline comet assay were used
- Leaf extracts from 4 different origins were investigated
- None of them showed *in vitro* toxicity or genotoxicity
- The extracts also didn't have antigenotoxic properties

Abbreviation list: BaP: benzo(α)pyrene, EMS: ethyl methane sulfonate, LMP: low melting point, NI_{50}: 50% inhibition of NRU, NR: neutral red, NRU: neutral red uptake, OD: optical density, PBS: phosphate buffer saline, SDS: sodium dodecyl sulphate, S/N: signal to noise ratio, 4NQO: 4-nitroquinoline oxide

Keywords: *Olea europaea L*, Vitotox test, comet assay, genotoxicity, antigenotoxicity

INTRODUCTION

Olive (*Olea europaea* L.) is an evergreen sclerophyllous tree cultivated in the Mediterranean region since ancient times. Olive orchards have been reliable producers of food and oil for thousands of years, supporting successive civilizations in the Mediterranean area. *Olea europaea* (syn. Zaytoun, in Arabic) belongs to the family Oleaceae and is a small evergreen tree, from 12 to 20 ft. high, with hoary, rigid branches, and a grayish bark. It is greatly growing in developed and developing countries for its known healing effects.[1] It is for example widely used in folk medicine in the European Mediterranean area, Arabia peninsula, India, and other tropical and subtropical regions, as diuretic, hypotensive, emollient, and for urinary and bladder infections.[2] Leaves are taken orally for stomach and intestinal diseases and used as mouth cleanser.[3] Decoctions of the dried fruit and of dried leaf are taken orally for diarrhea and to treat respiratory and urinary tract infections. Leaves decoction has also hypotensive,[4] antidiabetic,[5] anti-inflammatory,[6] diuretic,[7] and anticancer[8] activities. Despite its therapeutic value, this medicinal plant was never tested for its possible adverse health effects the way our modern pharmaceutical products are. It is important to investigate not only its potential adverse effects but also its potential beneficial effects. The aim of this study is therefore to fill this gap by investigating the genotoxic activity of four varieties of *Olea europea L* leaf extracts and to compare their activities.

MATERIAL AND METHODS

Plant material

Olea europea leaf varieties were selected from different regions in Tunisia, Chetoui (North), Meski (North), Oueslati (Center), Jarboui (Sahel). The varieties were identified by Professor Dalenda Boujnah from the olive institute of Sousse, Tunisia and voucher specimen numbers were attributed to each of the samples.

Preparation of leaves aqueous extracts

Three grams of dried material (leaves) was extracted by soaking in 100 mL distilled water at ambient temperature for 24 h in a shaker to give a concentration of 3% dry tissue. Extracts were then filtered in vacuum. First, through a Whatman #3 disk and then, re-filtered through a nitro-cellulose paper (Ø = 0.45 μm) to reduce the risk of interference by micro-organisms.

Genotoxicity test: bacterial Vitotox test

Two *Salmonella typhimurium* TA104 constructs were obtained as components of the Vitotox™ 10 Kit from GENTAUR bvba (Kampenhout, Belgium). They were grown overnight at 37°C (shaking) to obtain bacteria in the early logarithmic growth phase, corresponding to an approximate optical density (OD) of 0.5–0.6 at 590 nm. Cultures were then diluted and exposed to the test agent as described elsewhere.[9] The first construct contains a luciferase gene under control of the recN promoter, which results in light production when DNA is damaged (TA104-recN2-4 or genox strain). Light is measured with a luminometer (Modulus Microplate Multi-mode Reader, Turner Biosystems, Leiden, The Netherlands) every 5 min during 4 h. A signal-to-noise (S/N) ratio above 1.5 indicates genotoxicity.[9,10] The second strain contains the lux gene under control of a constitutive promoter so that the light production is not influenced by genotoxic compounds. This so-called TA104-pr1 strain (or Cytox strain) is used as an internal control. If light production goes down in this strain, it indicates a toxic response; if light production goes up it indicates that the test compound influences the lux gene other than via DNA damage. In this case, a "positive" response in the Genox strain probably does not reflect genotoxicity as could initially be thought. Bacteria were exposed to the extracts in different concentrations (0.02–0.1–0.5 mg/mL and 0.2–1.0–5.0 mg/mL) in the presence and absence of a metabolizing S9 fraction to investigate their genotoxicity. They were also treated with the extracts together with a chemical mutagen to investigate antigenotoxicity. Therefore, extracts were tested in the same concentrations as well as in three lower concentrations (1/5 dilutions). The chemical mutagens were 0.4 ppm 4-nitroquinoline oxide (4NQO; in the absence of S9) and 800 ppm benzo(α)pyrene (BaP; in the presence of S9). The mutagens were also used in the same concentration as positive controls in all tests. Bacteria and test compounds (extracts with or without mutagen) were then placed into the luminometer where light emission was measured (4 h). The culture medium was not changed in between. Therefore, extracts and chemical mutagens were still present in the medium when the cells were placed in the luminometer.

In vitro toxicity test: the neutral red uptake test

The NRU test[11] is based on the ability of living cells to take up and bind NR. NR is a dye which easily penetrates cell membranes via nonionic diffusion. It accumulates in the lysosomes. Xenobiotics acting on lysosomal membranes are responsible for a decreasing NR uptake. Living cells can therefore be distinguished from dead or dying cells based on their different NR uptake (NRU). We performed the NRU test according to well-known standard methods.[12] Cell suspensions of human C3A cells in Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum were seeded into each well of a 96-well microtiter plate such that the cell density was 40,000 cells/well. Plates were incubated overnight at 37°C and 5% CO_2. Humidity was maintained using a water bath containing milli-q water inside the incubator. After 24 h incubation, the cells were treated with dilutions of the extracts. Following another 24 h incubation time, cells were washed in PBS after which 200 mL of a 0.625 mg/mL NR-solution were added. Cells were washed 3 h later to remove excess of the dye. Then, 200 mL of a 50:1 ethanol-acetic acid solution was added to extract the dye from the cells. This was done in a microtiter plate shaker for approximately 1.5 h (until appearance of a homogenous purple color). Then, absorbance against a blank reference was measured at 540 nm using a microplate spectrophotometer. For all wells, OD values were calculated as the measured value minus the control value (V_c). Results were expressed as percentage of the OD determined from the average of the blank control culture read at 540 nm and set at 100%. The NI_{50} (50% inhibition of NRU) was determined from the dose–response curve of the mean OD values of the different concentrations. For the positive control, a separate plate was used where cells were treated with different concentrations of sodium dodecyl sulfate (SDS; 0-0.42 mM), and the NI_{50} was determined as for the herbal extracts described above. The NI_{50} should be within limits that were determined from 10 independent experiments from which average NI_{50} values, and standard deviations were calculated (unpublished data). The calculated NI_{50} for the positive control in an experiment should be within 2.5 SD of these historical data for SDS. If this is not the case, the results cannot be accepted, and the test should be repeated. The reported results were all in accordance with the requirements.

Genotoxicity test: comet assay

Possible DNA breakage effects were investigated by the alkaline comet assay on human C3A hepatic cells. The test was performed according to standard methods.[13] In short, cells were grown in 24-well plates (1 mL/400,000 cells). After a 24 h growth period, plant extracts were added in different concentrations. Concentrations were 5.0, 1.0, 0.2, and 0 mg/mL. Cells were trypsinized after another 24 h, brought to PBS and kept on ice to prevent further DNA damage. A 10 mL cell suspension + 300 mL 0.8% LMP agarose was brought on precoated slides (1% NMP agarose). Slides were kept on ice for 5 min and then brought in lysis buffer (2.5M NaCl; 100mM EDTA; 10mM TRIS; 1 v% Triton X-100 and 10 v% DMSO). The pH was adjusted to pH = 10 with NaOH pellets. The slides remained overnight into the lysing solution. The next day, slides were brought into denaturation buffer (0.3M NaOH, 1 mM EDTA in water, t = 17°C, pH = 13) in which electrophoresis (20 min, 1.2 V/cm, 300 mA) occurred. After lysis, histones and nucleosomes were removed leaving supercoiled DNA behind. DNA damage results in broken DNA fragments and loops that will unwind and migrate in the agarose gel. A “comet-like” figure is formed that can be visualized after staining with a fluorescent dye. Slides were therefore dried, renaturated in 200 mL H_2O (10 min) and stained for another 10 min with 100 mL gel red (1:3300 stock solution). Afterward, slides were analyzed with an Axio Imager. Z2 (Zeiss) fluorescence microscope with Metacyte and Metafer 4 (version 3.8.5) software from Metasystems (Altlussheim, Germany). The percentage DNA in the comet tail was used as the measure of DNA damage. Ethyl methane sulfonate (0.75 mM) was included as a positive control. Two slides were prepared per exposure, and a total of 100 cells (DNA comets) were measured (50 per slide). The Mann–Whitney U test was used to determine statistical deviations from the unexposed control cells.

RESULTS AND DISCUSSION

Most of the traditional medicinal plants have never been the subject of exhaustive (geno) toxicological tests such as is required for modern pharmaceutical compounds. On the basis of their traditional use for long periods of time, they are often assumed to be safe. But research has shown that a lot of plants with recognized beneficial properties and which are used as food ingredients or in traditional medicine can yet also be mutagenic or toxic or even carcinogenic.[14,15,16] Genotoxic plants should be considered potentially unsafe and certainly require further testing before their continued use can be recommended. Plants with obvious antigenotoxic potential can, on the other hand, be considered interesting for therapeutic use and merit further in depth investigations of their pharmacological properties. This is why our research on olives (*Olea europaea* L.) also includes a (geno) toxicological part which is reported here. For the investigation of the potential genotoxicity/antigenotoxicity of the plant extracts, we used two indicator tests, the bacterial Vitotox test and the alkaline comet assay in human C3A cells. We chose both tests as previous investigations on a large number of medicinal plant extracts from different origin showed that a combination of both test is usually sufficient to decide upon an extract's genotoxicity profile. Therefore, the use of more time-consuming tests is apparently not imperative.[17]

The Vitotox test was at first performed in concentrations of 0.02–0.1–0.5 mg/mL. This corresponds to a dose range that was found accurate in many similar experiments with plant extracts.[18,19] Then, because of lack of any effect, also higher concentrations of 0.2–1.0–5.0 mg/mL were studied. The concentration of 5 mg/mL corresponds with the limits of solubility. Figure 1 gives an example of the results obtained for the extract “Meski” in the absence and presence of S9. It can be seen that in cultures without addition of S9 no toxicity was found (S/N ratio in Cytox strain remains approximately “1”) and that there was no genotoxicity as S/N in the Genox strain also did not increase. In the presence of S9 there is a slight increase in S/N in both the Cytox (up to S/N $\cong$ 1.2) and Genox strain (S/N reaching levels just below 1.5) but the criteria for genotoxicity (i.e., S/N > 1.5) were not yet fulfilled. Repeat experiments gave the same result (slight increase but at the most just below the threshold for genotoxicity). Another representation of the results is given in Figure 2 (for all extracts). It can be seen that the positive controls (4NQO and BaP) always showed genotoxicity (S/N > 1.5 in the Genox strain) and no toxicity (S/N >0.8 in the Cytox strain). The tested extracts where not toxic but also not genotoxic according to the Vitotox test criteria. Borderline genotoxicity was yet found for the Meski sample in the present of S9 (as indicated before). The same holds true for the Ouslati sample (both at the maximum concentration of 5 mg/mL) but the conclusion that none of the samples was genotoxic remains valid yet.

The comet assay was performed on human C3A cells that retain their metabolic activity. For this reason tests were only performed in the absence of S9. Investigated concentrations were the highest possible (5 mg/mL, solubility limit concentration and highest recommended dose[20]) and two further dilutions from this. All tested doses were found nontoxic in the NRU test (NI_{50}>90%; Figure 3). Figure 3 is representative for all four extracts as none of them had NI_{50} values lower than 90%. The comet assay was therefore performed on nontoxic (but yet high) concentrations of the extracts [Figure 4]. Statistically significant deviations were found at 1 mg/mL for the Chetoui sample but this was borderline and a higher concentration did not show increased DNA damage. Extract Meski did show increased DNA damage (% tail DNA) at the two highest concentrations. Taking the results of the Vitotox test and comet assays together we can conclude that only the Meski extract may have some genotoxic properties. However, the effect was low as can be seen when it is compared to that of well-known mutagens. Genotoxicity was, for example, much less impressive than that of the positive controls EMS (approximately 30% tail DNA at 0.75 mM), or 4NQO where concentrations of 0.075 up to 0.25 μg/mL showed highly significant increases of DNA damage ($P < 0.001$, results not shown).

Antigenotoxicity was also investigated in the Vitotox test. Here, the same extract concentrations and three further dilutions were tested in the presence of 4NQO or BaP. Figure 5 shows the results for the concentrations 0.2–1.0–5.0 mg/mL. Concentration “0” corresponds to 4NQO alone (without addition of an extract), concentration 0.2 mg/mL means that 4NQO was tested in conjunction with 0.2 mg/mL extract, etc. The same holds true for the samples with S9 where “0” is BaP alone and 0.2 is BaP + 0.2 mg/mL of the extract. Figure 5 shows that the extracts do not significantly reduce (or enhance) the genotoxicity of the known mutagen and hence that the extracts apparently do not have a strong antigenotoxic (or cogenotoxic) activity. As antigenotoxicity may be concentration dependent we also investigated lower concentrations of the extracts (1.6–8.0–40 μg/mL). The results did not show any important deviation from the damage induced by the known mutagens (not shown). We thus conclude that the four olive extracts (*Olea europaea* L.) obtained from different regions in Tunisia are not genotoxic (although some doubt exist with respect to the extract collected at Meski). None has important antigenotoxic properties and also do not enhance the genotoxicity of the mutagens 4NQO or BaP. Lack of antigenotoxicity, may indicate that the previously reported anticancer effects[8] do not result from protection against genotoxicity. The negative genotoxicity data furthermore underline the safe use of the leaves, for example, in hypoglycemic and antidiabetic preparations.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Figures and Tables

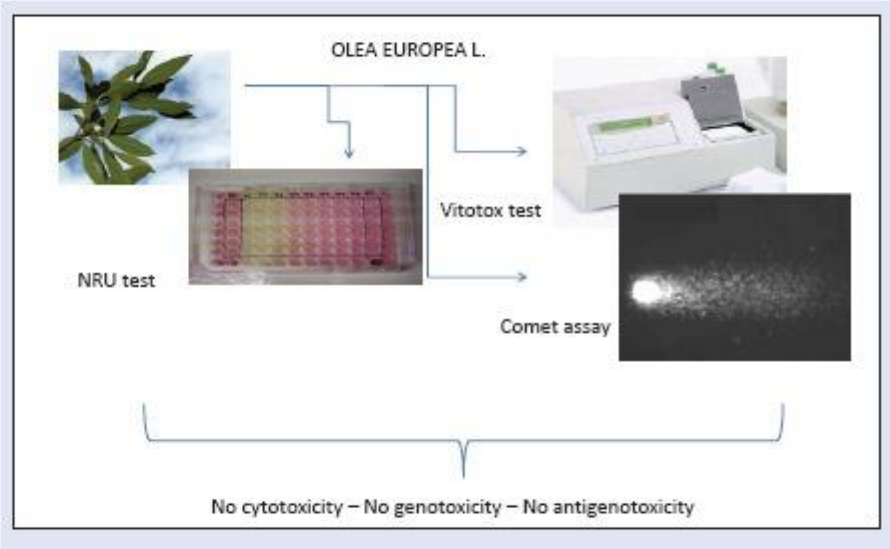
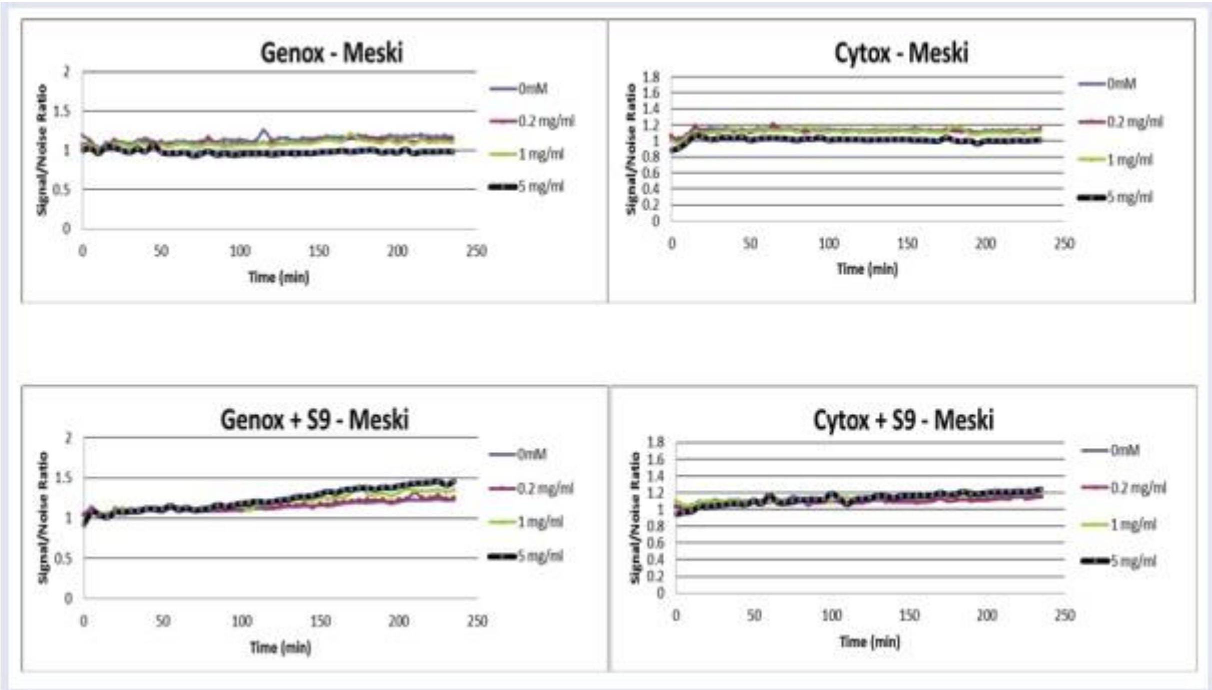
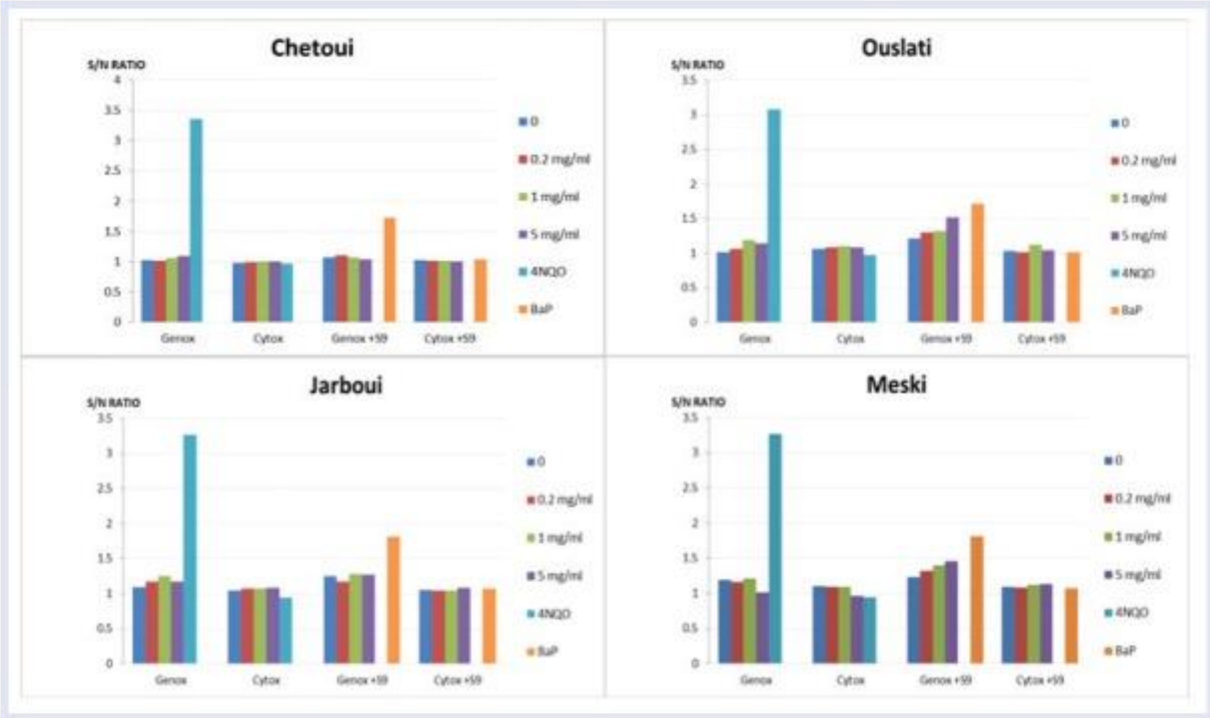


Figure 1



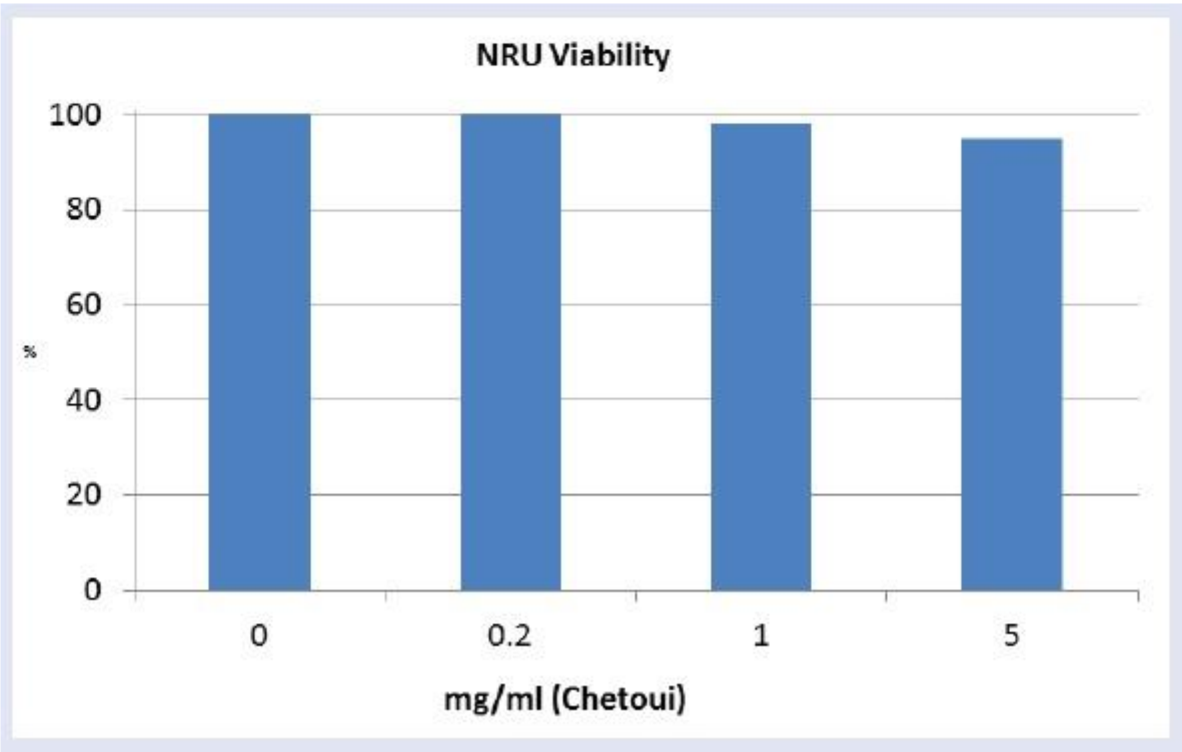
Example of Vitotox test results for one of the investigated extracts (Meski) in the absence and presence of S9.

Figure 2



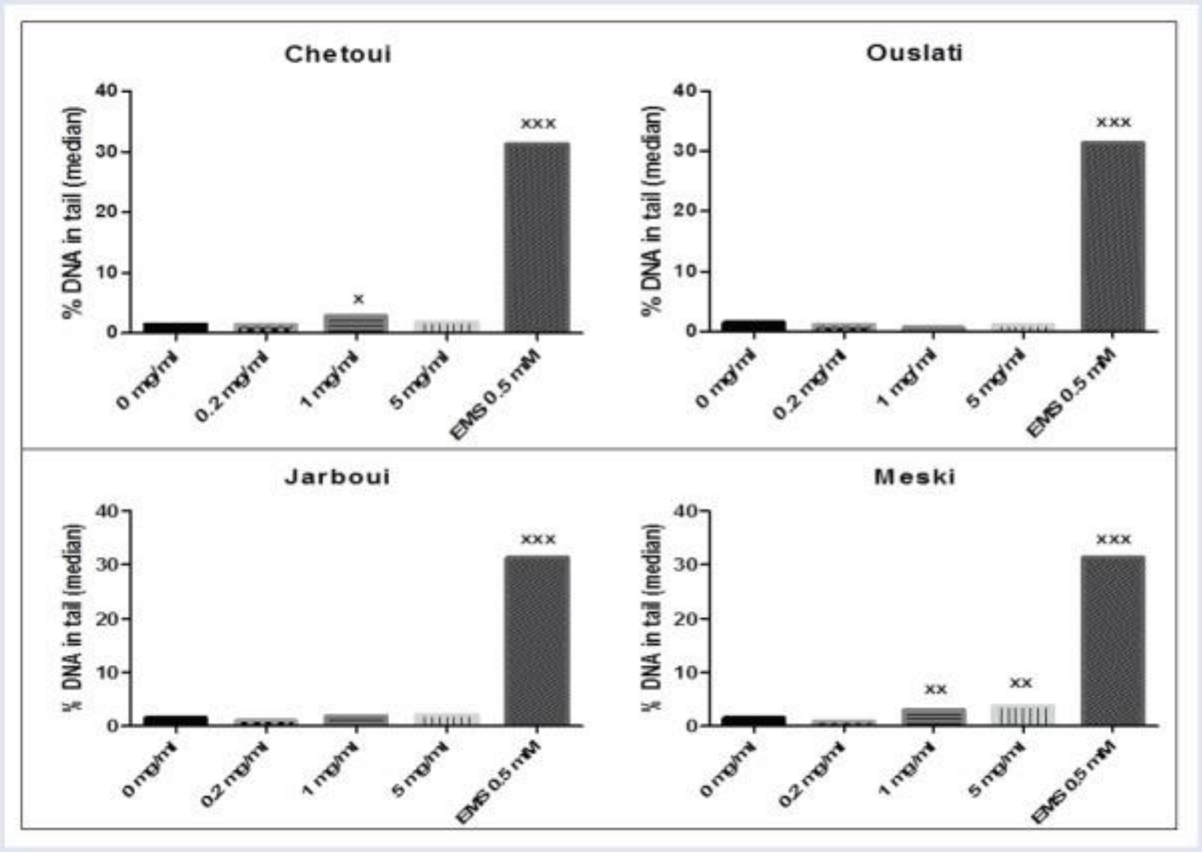
Representation of Vitotox test results for all four extracts in the presence and absence of S9.

Figure 3



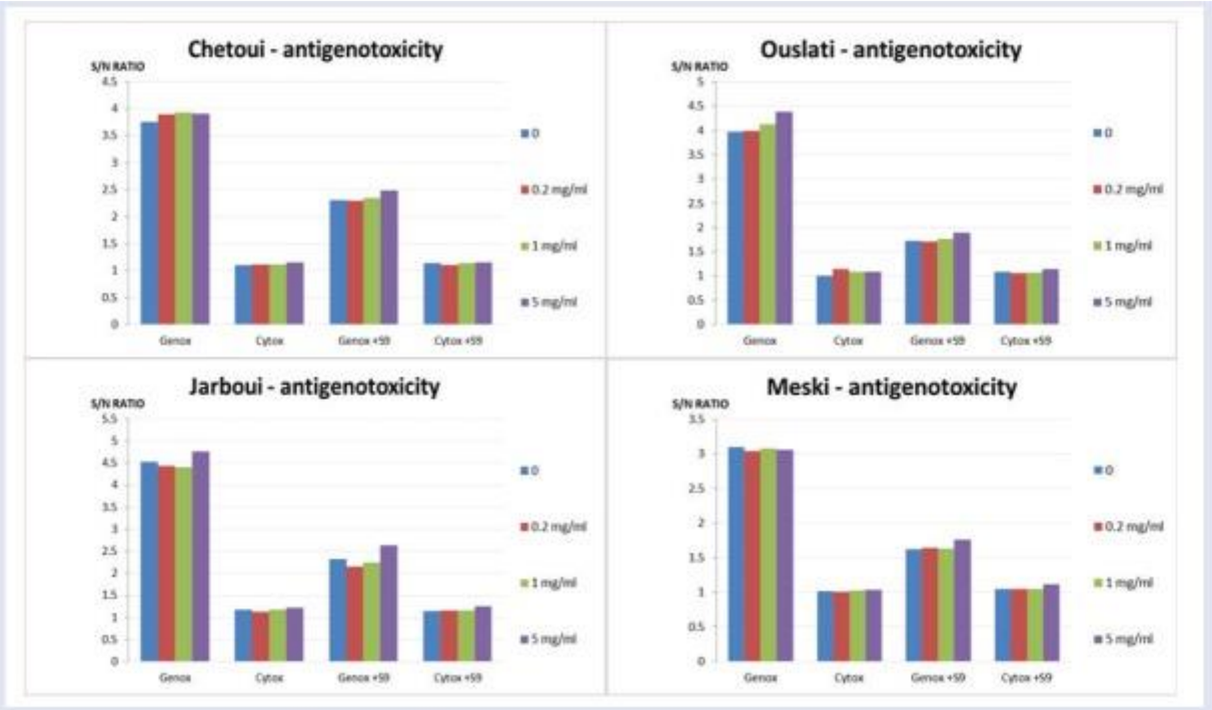
Example of NRU test results for the extract from Chetoui.

Figure 4



Comet test results for all four extracts. Statistical significant increases above background levels were indicated as x = $P < 0.05$; xx = $P < 0.01$, and xxx = $P < 0.001$.

Figure 5



Vitotox test results on the possible antigenotoxicity of the four olive extracts.

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