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Toxic and mutagenic properties of extracts from Tunisian traditional medicinal plants investigated by the neutral red uptake, VITOTOX and alkaline comet assays

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Abstract

We investigated the genotoxic properties of a number of extracts from Tunisian traditional medicinal plants with the bacterial VITOTOX test in *Salmonella typhimurium* and the alkaline comet assay in human C3A cells. Ethyl acetate and methanol extracts from *Marrubium alysson* L. and *Retama raetam* (Forsk.) Webb and methanol extracts from *Peganum harmala* L. were investigated. Toxicity was furthermore studied with the neutral red uptake test that served for dose-finding.

All extracts showed antigenotoxic properties against 4-nitroquinoline-oxide (4-NQO) and benzo(α)pyrene in the VITOTOX test, except the methanol extracts from *R. raetam* where antigenotoxicity was not found against the mutagen 4-NQO (in the absence of S9). The ethyl acetate extract from *R. raetam* was found mutagenic with the VITOTOX test in the absence of S9, whereas both ethylacetate and methanol extracts of *M. alysson* L. induced DNA damage according to the alkaline comet assay in C3A cells.

Keywords: Comet test; *Marrubium alysson* L.; NRU test; *Peganum harmala* L.; *Retama raetam* (Forsk.) Webb; Tunisian medicinal plants; VITOTOX test

1. Introduction

At present, unwanted side effects or loss of efficiency in the long run are the major limitations of existing medication. For this reason there is a continuous search for new medical preparations against a great number of ailments, including cancer. The search for inhibitors of mutagenesis may be useful as a tool to discover anticarcinogenic agents. On the other hand most of the traditional medicinal plants have never been the subject of exhaustive toxicological tests such as is required for modern pharmaceutical compounds. Based on their traditional use for long periods of time they are often assumed to be safe. However, research has shown that a lot of plants which are used as food ingredients or in traditional medicine have *in vitro* mutagenic (Cardoso et al., 2006; Deciga-Campos et al., 2007; Mohd-Fuat et al., 2007) or toxic and carcinogenic (De Sa Ferreira and Ferrao Vargas, 1999) properties. Within this context, it is also important to screen medicinal plants for their mutagenic properties. Plants exhibiting clear mutagenic properties should be considered as potentially unsafe and certainly require further testing before their continued use can be recommended. Plants with obvious antimutagenic potential can, on the other hand, be considered interesting for therapeutic use and merit further in depth investigations of their pharmacological properties.

In this paper we report on the results from the neutral red uptake (NRU), VITOTOX and alkaline comet assays that were applied to a number of plants used in traditional Tunisian

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medicine. The NRU test (Repetto et al., 2008) was essentially used to assess *in vitro* toxicity in the C3A cell line that we used and hence it served as a 'dose-finding' test. The VITOTOX test is a very sensitive genotoxicity reporter assay in *Salmonella typhimurium* bacteria based on SOS-response (Verschaeve et al., 1999). The (alkaline) comet assay detects single and double DNA strand breaks and has proven to be very interesting for many different applications, including product testing and animal and human biomonitoring studies (Liao et al., 2009).

The investigated plants were *Retama raetam*, *Peganum harmala* L. and *Marrubium alysson* L. *R. raetam* is used to treat diabetes as extracts of this plant are assumed to inhibit glucose absorption in the kidneys (Maghrani et al., 2005). Extracts from the flowers also seem to possess antibacterial, antiviral and antioxidant properties which resulted in their proposed use as a food preserving additive (Edziri et al., 2008, 2010). *P. harmala* is used in the treatment of many different ailments. Seeds are for example used to treat cancer. They contain harmaline and harmine that are topoisomerase I inhibitors (Sobhani et al., 2002). Both substances are also present in the roots and induce hallucinations due to inhibition of amine neurotransmitter metabolism (Frison et al., 2008). Extracts from the seeds have antibacterial activity (Arshad et al., 2008). Methanol extracts were shown to influence the expression of different P450 enzymes (increased expression of CYP1A2, CYP2C19 and CYPP3A4, and decreased expression of CYP2E1, CYP2B6 and CYP2D6; El Gendy and El-Kadi, 2009). All plant parts of *M. alysson* L. are used in traditional medicine for the treatment of hypertension and rheumatics. Preparations from flowers have laxative properties and petroleum ether extracts of the plant have antibacterial and antifungal properties (Edziri et al., 2007). However, little is known about the (geno)toxic effects of all these plants.

2. Materials and methods

2.1. Plant materials

All plants, *M. alysson*, *R. raetam* and *P. harmala* were collected in 2009 from Kerker (Tunisia). The plants were identified by Pr. Mohamed Chaieb, botanist at the University of Science of Sfax (Tunisia). The voucher specimens of *M. alysson* (MA 287), *P. harmala* (PA 068) and *R. raetam* (RE 207) are deposited at the Herbarium of the Faculty of Pharmacy of Monastir. Plants that were tested are listed in Table 1. Tests were performed with coded extracts that were designated as MP (methanol extract of *P. harmala* L.), MR (methanol extract of *R. raetam*), MM (methanol extract of *M. alysson* L.), AM (ethylacetate extract of *M. alysson* L.) and AR (ethylacetate extract of *R. raetam*). The code was broken only after all results were obtained.

2.2. Plant extraction

Powdered plant tissues (700 g) were extracted three times by maceration with methanol, the resultant extract was concentrated under reduced pressure. The methanol extract was extracted successively with equal volumes of organic solvents of increasing polarity i.e., chloroform, ethyl acetate and butanol. The different extracts were concentrated to dryness and kept at 4 °C.

2.3. The neutral red uptake (NRU) test

The NRU test measures cell viability based on the property of living cells to be able to take up neutral red dye into their lysosomes (Repetto et al., 2008). Dying cells have altered membrane properties and therefore they cannot anymore take up neutral red (NR). The dye is applied to cells in different concentrations allowing the determination of a NI_{50} concentration (50% reduction of uptake) by measuring OD_{540}.

This test was performed according to well known standard methods. Cells were plated in 96 well plates (40,000 cells per well) and incubated in DMEM + 10% FBS for 24 h at 37 °C and 5% CO_2. Plant extracts were then added in different concentrations for another 24 h. Cells were then washed with PBS after which 200 μL of a 0.625 μg/mL neutral red solution was added. After 3 h cells were again washed in PBS to remove the remaining dye. Addition of 200 μL ethanol/acetic acid (50/1) resulted in release of the dye from the cells that were placed in a shaking bath until a homogenous colour was formed (approx. 1 h). The optical

Table 1
Tunisian medicinal plants that were investigated for genotoxic and antigenotoxic (antimutagenic) properties.

Plant species	Medicinal use	Part used	Extract with coded name (into brackets)	Weight tube+ extract (g)	Weight tube (g)	Weigth extact (g)	Concentration stock solution (mg/mL)	NRU NI_{50} (mg/mL)	Range tested (mg/mL)
Marrubium alysson L.	Hypertension Cough Burns Rheumatics Intestinal troubles	Aerial parts	Ethylacetate (AM)	3.2749	3.1078	0.1671	180	0.101	0.03–0.06
			Methanol (MM)	3.6156	3.1646	0.4510	300	Not possible (highest concentration >50% viability)	0.5–2.0
Retama raetam	Hypertension Diabetes	Flowers	Ehtylacetate (AR)	3.3271	3.1275	0.1996	180	0.040	0.005–0.02
			Methanol (MR)	3.4625	3.1245	0.3380	300	1.171	0.15–0.6
Peganum harmala L.	Cancer Fever Emmenagogue lactogogue	Aerial parts	Methanol (MP)	3.6023	3.1784	0.4239	300	Not possible (highest concentration >50% viability)	2–3

density was measured with a spectrophotometer. The OD_{620} measured as a reference value was subtracted from the OD_{540} which is the optical density at the wavelength at which maximal absorption of NR occurs. Absorption of non-treated cells was given a 100% value to which data from exposed cells were compared. SDS (sodium dodecyl sulfate) was used as a positive control.

2.4. The VITOTOX test

The VITOTOX test was used to investigate the potential mutagenicity of the plant extracts as well as their possible antigenotoxic properties against the well known mutagens 4-nitroquinoline-oxide (4-NQO) and benzo(α)pyrene. Concentrations used were as indicated in Table 1 and based on preliminary dose-finding and toxicity tests using the NRU assay. A detailed description of the VITOTOX test is given elsewhere (Verschaeve et al., 1999; Verschaeve, 2005). Shortly, the VITOTOX test can be used as a simple largely automated high throughput genotoxicity test. In this test two different *S. typhimurium* TA104 constructs are used. One contains a luciferase gene under control of the recN promoter which results in light production when DNA is damaged (TA 104-recN2-4 strain or *Genox* strain). A sample is considered genotoxic when the signal to noise ratio (sample/unexposed control) increases and reaches levels above 1.5. 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 this is indicative of a toxic response, if light production goes up this indicates that the test compound influences the *lux* gene in another way than via DNA damage. In this case a 'positive' response in the *Genox* strain most probably does not reflect genotoxicity as could initially be thought.

Bacteria were cultivated in 96 well plates and then transferred to a luminometer in the presence and absence of a rat metabolic enzyme fraction (S9) and the appropriate test compounds. Light measurements were performed in a luminometer at 30 °C. This was done every 5 min in each well during a 4 h period. The direct acting base-alterating mutagen 4-NQO and the indirect mutagen benzo(α)pyrene which requires metabolic activation were used as positive controls. Antimutagenicity was investigated against the same mutagens.

2.5. The alkaline comet assay

The test was performed according to standard methods (Olive and Banáth, 2006). In short, C3A cells were grown in 24-well plates (1 mL/400000 cells). After a 24 h growth period plant extracts were added in different concentrations. Concentrations were chosen based on the results of the NRU test. Cells were trypsinized after another 24 h, brought in PBS and kept on ice to prevent further DNA damage. A 10 μL cell suspension + 300 μL 0.8% LMP agarose was brought on pre-coated slides (1% NMP agarose). Slides were kept on ice for 5 min and then brought in lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM 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 (0.3 M NaOH, 1 mM EDTA in water, t = 17 °C, pH = 13) in which electrophoresis (20 min, 1 V/m, 300 mA) occurred. After lysis, histones and nucleosomes were removed leaving super-coiled 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 μL H_2O (10 min) and stained for another 10 min with 100 μL gelred (1:3300 stock solution). Afterwards, slides were analysed with an Axio Imager.Z2 (Zeiss) fluorescence microscope with Metacyte and Metafer4 (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 at least 100 and often more than 200 cells (DNA comets) were measured, evenly distributed over the two slides. C3A cells are derived from HepG2 cells. They retain many of the properties of the normal human hepatocyte. They have the essential structural, biochemical and growths features of normal human liver cells and have conserved both phase I and phase II metabolic capacities (Kelly, 1994). For this reason the comet assay was performed in the absence of S9 only.

2.6. Statistical analysis

No statistics were needed for the NRU test where we only determined NI_{50} values and the VITOTOX test were a dose–effect relationship and S/N ratio (Genox over Cytox strain) reaching levels over 1.5 are sufficient to decide about a compound's genotoxicity. The Mann–Withney *U*-test was used for analysis of comet test data.

3. Results

The results of the neutral red uptake test are summarized in Table 1 where NI_{50} concentrations are given. For the methanol extract of *M. alysson* L. and *P. harmala* L. NI_{50} concentrations could not be determined since viability was still higher than 50% at the highest tested concentration.

Fig. 1 gives an example of the results that were obtained with the positive controls (4 μg/L 4-NQO and 8 μg/mL benzo(α) pyrene) that were also used in the combined exposure experiments (antigenotoxicity testing) with the VITOTOX test. No significant deviation from S/N = 1 was found in the cytox strain indicating that there was no cytotoxicity nor direct influence of the chemical upon the lux operon. The genox strain showed increased light production reaching levels well above S/N = 1.5 indicating genotoxicity. All experiments were accompanied with their own negative and positive controls that

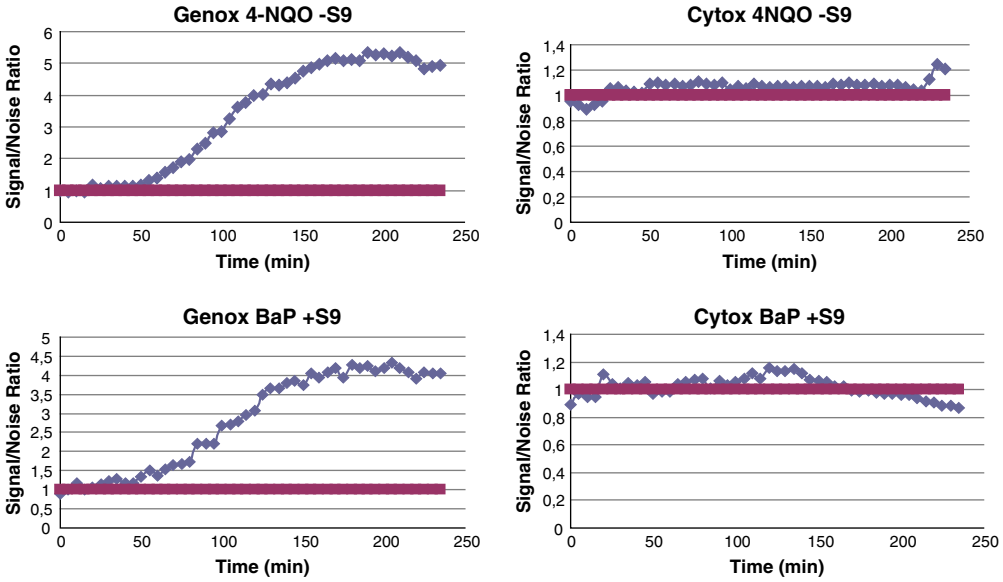

Fig. 1. VITOTOX® results for the positive controls 4-NQO (without S9) and benzo(α)pyrene (with S9). The Genox strain clearly shows that both chemicals are genotoxic (S/N > 1.5) whereas the Cytox strain shows no significant deviation from S/N = 1 and hence absence of toxicity or a direct (DNA damage unrelated) influence of the lux operon.

showed the expected results. An overview of all results is given in Table 2 and illustrated for a few examples in Fig. 2.

Methanol extracts from *P. harmala* L. (MP, not shown) without S9 showed decreased light production in the Cytox strain at all concentrations tested. A similar decrease was observed in the Genox strain. Therefore we may conclude that there was no indication of genotoxicity. There was also no indication of genotoxicity in the presence of S9. Combined treatment with the mutagens also showed decreased light production in the Cytox strain (possibly indicating toxicity) but decreases compared to the mutagens alone were more important in the Genox strain. This indicates that the extract lowers the mutagenicity of the well known mutagens used in this study. The extract is therefore to some extent antigenotoxic.

The results are similar for the MR extract (Methanol extract of *R. raetam*). There were no signs of genotoxicity but there was again some toxic response as indicated by decreased light production in the Cytox strain. Decreased light production for the combined MR + 4-NQO treatment compared to 4-NQO treatment alone at first sight indicates antigenotoxicity. Nevertheless, as this decrease was also present at more or less the same extent in the Cytox strain this extract apparently does not protect against the mutagenicity of 4-NQO (Fig. 2). It is antigenotoxic against benzo(α)pyrene in the presence of S9 (not shown).

The methanol extract of *M. alysson* L. (MM), was not genotoxic. However, in the presence of S9 we found increased light production (above S/N = 1.5) in both the Cytox and Genox strains (Fig. 2). This indicates that the extract directly influences the Lux operon (light production not induced by DNA damage). The extract is therefore not genotoxic. It showed some antigenotoxic response against both mutagens. This is illustrated in Fig. 2 where combined effects with 4-NQO in the absence of S9 are presented. The Cytox strain shows decreased light production compared to 4-NQO-treated bacteria alone but the decrease is much more important in the Genox strain. Lower mutagenicity is consequently not only due to some toxic response.

The ethyl acetate extract of this plant (AM) was obviously not genotoxic, both in the presence and absence (Fig. 2) of S9. Combined treatments again showed some toxic response (decreased light production below S/N = 0.8) but this cannot explain the more important reduction in light production in the genox strain (compared to the mutagens alone). Therefore AM was found antigenotoxic, especially against 4-NQO. Antimutagenicity against benzo(α)pyrene was less obvious.

Table 2
synthesis of the results obtained in the VITOTOX test; −S9/+S9: plant extracts alone in the absence or presence of S9; + 4-NQO/benzo(α)pyrene:plant extracts in combination with the mutagens.

Extract	−S9	+S9	+ 4-NQO (−S9)	+ benzoα)pyrene (+S9)
MP — methanol extract of *Peganum harmala L.*	−	−	Antigenotoxic	Antigenotoxic
MR — Methanol extract of *retama raetam*	−	−	−	Antigenotoxic
MM — Methanol extract of *Marrubium alysson L.*	−	−	Antigenotoxic	Antigenotoxic
AR — Ethylacetate extract of *retama raetam*	+	−	Antigenotoxic	Antigenotoxic
AM — Ethylacetate extract of *Marrubium alysson L.*	−	−	Antigenotoxic	Antigenotoxic (weak)

Contrary to the other extracts we found a clear dose-dependent genotoxic action for the ethyl acetate extract of *R. raetam* (AR) in the absence of S9 (Fig. 2). With S9 there was no genotoxicity. The extract was found antigenotoxic against both chemical mutagens. All results were confirmed by a repeat study that had exactly the same outcome (results not shown).

Comet assay results are presented in Fig. 3. The % DNA in the comet tail is given as a measure of DNA damage which, in our hands and according to others (Kumaravel and Jha, 2006), is the most suitable parameter. The positive control used in all our experiments (0.75 mM ethyl methane sulfonate) always showed the expected result (% DNA in the comet tail varied between 50

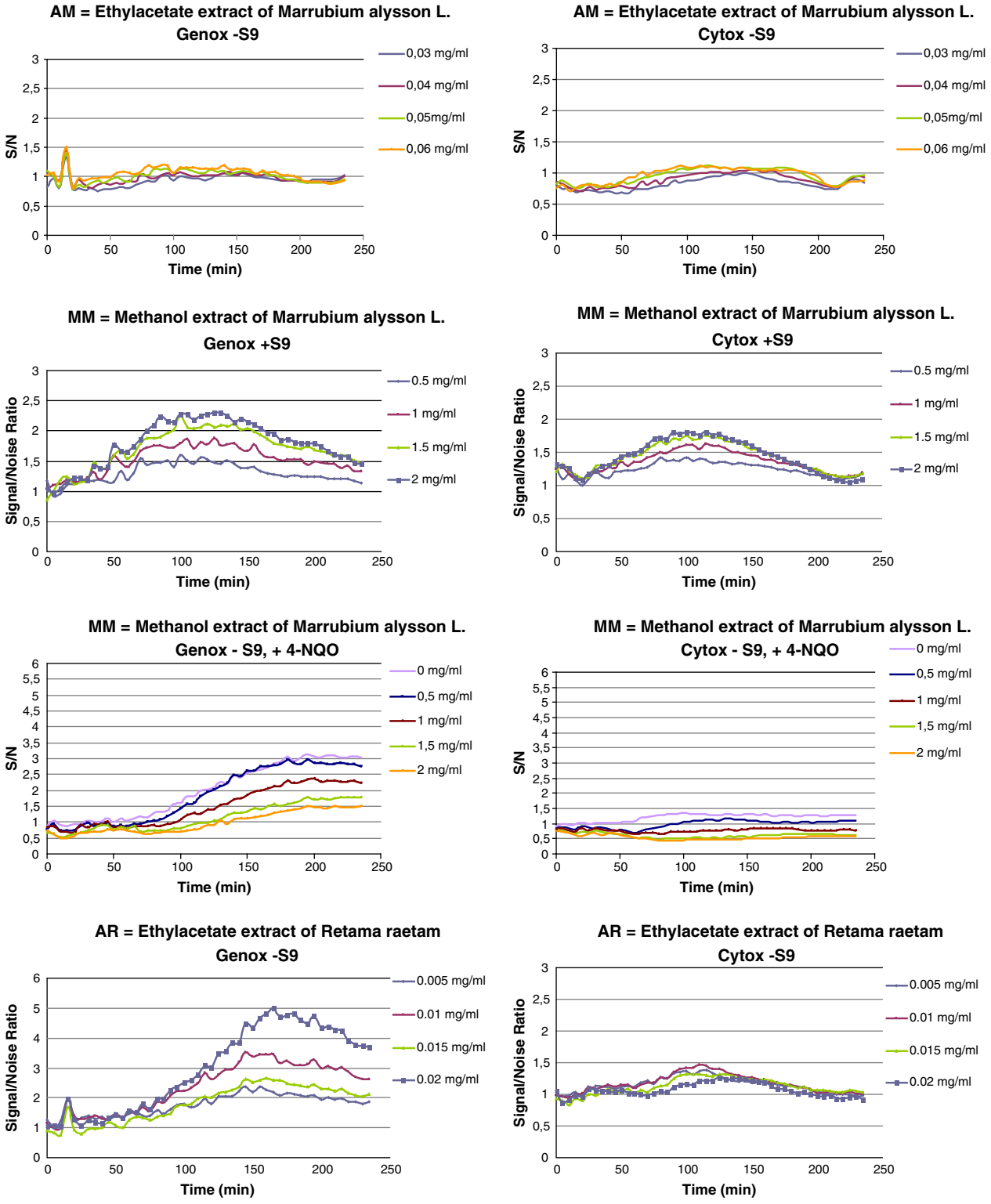

Fig. 2. Some examples of VITOTOX results. All experiments were performed twice. Repeat experiments (not shown) gave the same results.

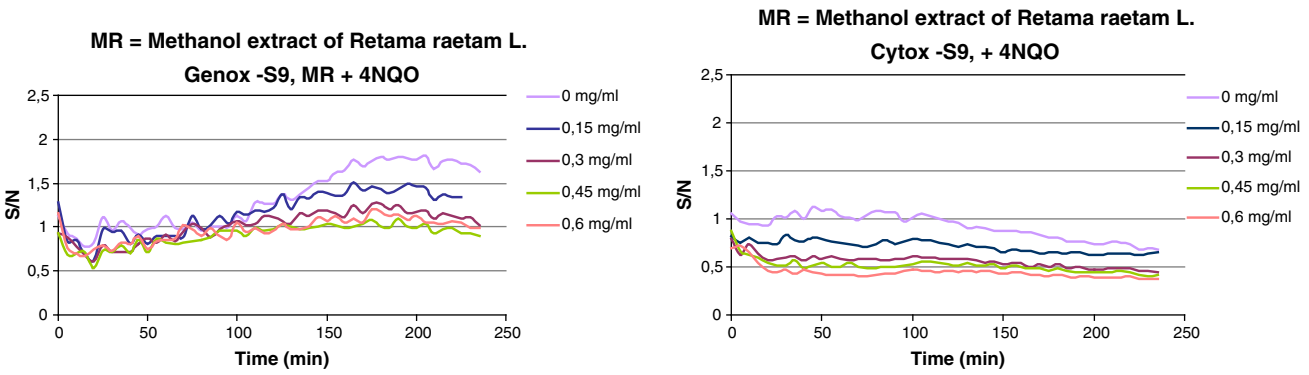

Fig. 2. (*continued*).

and 75% according to the experiment). Significant, though much lower DNA damage was also found with virtually all tested concentrations of the ethylacetate extract of *M. alysson* L. DNA damage was also significantly increased with 1 mg/mL of the methanol extract. Higher doses did not show DNA damage but this may partially be ascribed to increased toxicity that was obvious at 2 mg/mL (NI_{50} = 58.8%). Other extracts (*R. raetam* and *P. harmala* L.) were not found to damage DNA. The AR extract was only investigated in two concentrations (0.015 and 0.02 mg/mL). Intermediate concentrations of *R. raetam* methanol extracts did show reduced DNA damage compared to the unexposed controls which was statistically significant at 0.3 mg/mL ($p < 0.05$).

4. Discussion

The VITOTOX test was found very suitable and highly efficient for (high throughput) screening of chemicals to determine their genotoxic potential (Benfenati et al., 2009; Muto et al., 2006; Verschaeve, 2005; Westerink et al., 2009). The test is however less efficient when complex mixtures, e.g., concentrates from surface waters or pollutants extracted from air filters are studied (Verschaeve, 2005). Often decreased light production is found (especially in the Cytox strain) which can be interpreted as a toxic response and this of course may to some extent complicate the evaluation of genotoxic and/or antigenotoxic properties. Plant extracts are also complex mixtures and therefore the VITOTOX

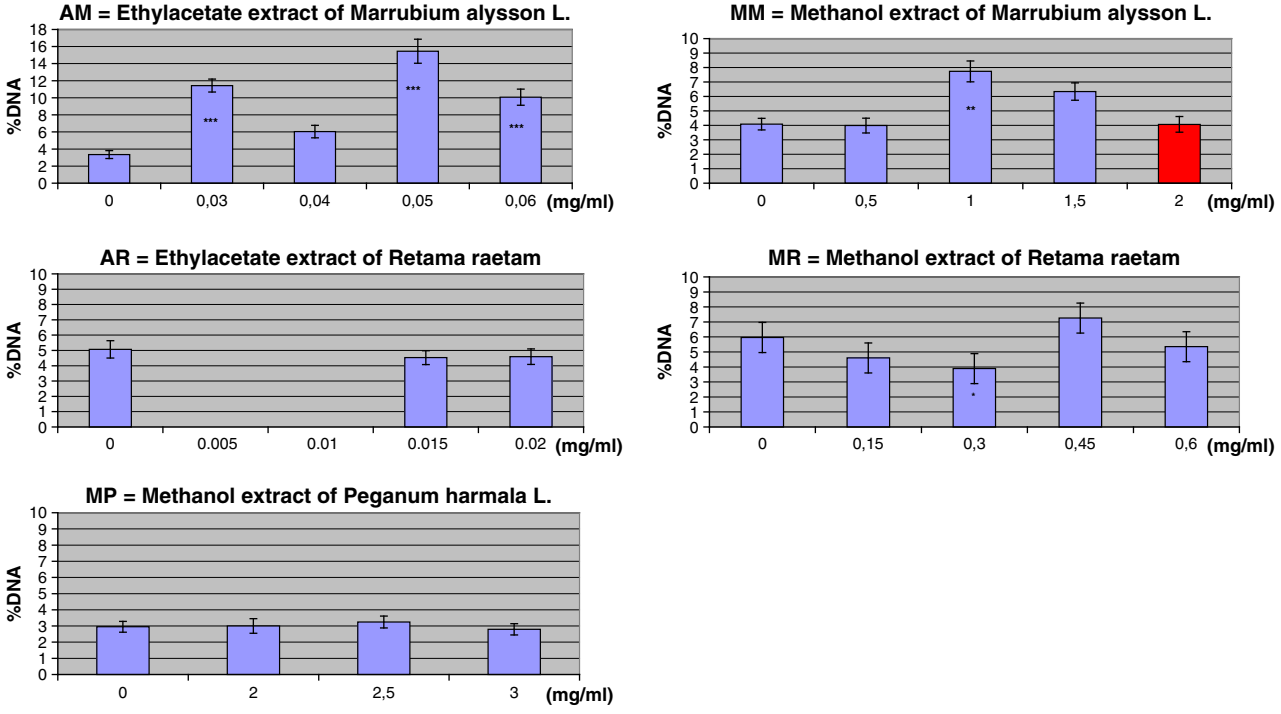

Fig. 3. Comet test results. Statistical significant differences from the untreated controls is given by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). 2 mg/mL of the MM sample was toxic (NI_{50} = 58.8%).

test is probably not the best test to investigate their genotoxicity. We nevertheless previously demonstrated that it can be used for such samples and be valuable for screening large numbers of samples (Verschaeve and Van Staden, 2008). The test was therefore applied to these extracts as a *first screening* of their mutagenic and antimutagenic properties. We often found some signs of toxicity (decreased light production) as we found for other complex mixtures and this for concentrations that were subtoxic to nontoxic in the NRU assay (not shown). We yet were able to show that one (AR) out of five extracts is mutagenic (genotoxic) in the absence of S9 only. It nevertheless is antigenotoxic against 4-NQO. The extract may therefore be an example of a so-called Janus-compound as it apparently shows mutagenic as well as antimutagenic properties (Bhattacharya, 2011; Von Borstel and Higgins, 1998). All extracts were found to be to some extent antigenotoxic, except MR where antimutagenicity against 4-NQO was not found. Despite the complex nature of the samples antigenotoxicity could thus also be detected, although confirmation by other tests may be required. Antigenotoxicity of plant extracts was shown on many occasions and it is therefore not surprising that most of our extracts also belonged to the group of antigenotoxicants. We previously, for example, found pronounced antigenotoxic properties of dichloromethane and methanol extracts of *Bauhinia galpinii* as well as of *Rotheca myricoides* extracts that proved to be most promising in terms of their antimutagenicity (Verschaeve and Van Staden, 2008). In another paper antimutagenicity was found for a number of extracts from vegetables with higher antimutagenic properties of organically vs. conventionally cultivated vegetables (Ren et al., 2001). Many other examples are found in the scientific literature.

From the 5 extracts that were tested the VITOTOX test thus showed that only the ethylacetate extract from *R. raetam* (AR) was mutagenic. It apparently is a direct mutagen (active without S9) which loses its mutagenicity after metabolisation (not mutagenic in the presence of S9). This can be the reason why it did not damage DNA in C3A cells as shown in the comet assay. On the other hand we found that methanol and especially ethyl acetate extracts of *Marubium alysson* L. damaged DNA according to the comet assay. This was not found in the VITOTOX test, presumably indicating that DNA damage is induced by a mechanism that is not detected in the bacterial assay. Westerink et al. (2009) have compared the VITOTOX test with the RadarScreen assay in yeast and concluded that, contrary to the RadarScreen assay, the VITOTOX test shows a high correlation with the Ames test. The VITOTOX test also detects some clastogens (chromosome breaking) chemicals, but usually only Ames-negative compounds. The RadarScreen cannot be used to predict Ames test results, but it predicts clastogenesis quite well. This illustrates that different genotoxicity tests may have different specificities and sensitivities against certain types of mutagens and that an individual test does not detect all mutagens. This is the reason why more than one, and especially complementary genotoxicity tests are required. The VITOTOX and comet assays may be such tests.

In conclusion, our results show that both *R. raetam* (AR) and *M. alysson* L. (AM and MM) have some genotoxic properties that require further investigation. They may furthermore be Janus-mutagens as they also show some antigenotoxic properties.

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