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Comparison of phenolic and volatile profiles of edible and toxic forms of *Detarium senegalense* J. F. GMEL

Nafissatou DIOP NDIAYE[1]*, Sylvie MUNIER[2], Yves PELISSIER[2], Frédéric BOUDARD[2], Christian MERTZ[3], Marc LEBRUN[3], Claudie DHUIQUE-MAYER[3] and Manuel DORNIER[4]

[1]Institut de Technologie Alimentaire, Route des Pères Maristes, B.P. 2765, Dakar - Hann, Sénégal.
[2]Université Montpellier I, UFR Pharmacie, UMR95 QualiSud, 15 av. Charles Flahault, F-34090 Montpellier, France.
[3]Cirad, UMR95 QualiSud, 73 rue J.F. Breton, TA B-95/16, F-34398 Montpellier cedex 5, France.
[4]Montpellier SupAgro, UMR95 QualiSud, 1101 av. Agropolis, B.P. 5098, F-34093 Montpellier cedex 5, France.

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In Senegal, *Detarium senegalense* J.F. Gmel. (ditax in Wolof) is one of the most important forest fruit species. However, exploitation of the edible fruit is based on local people's knowledge. Only trees whose fruits are consumed by animals are exploited. To identify them, a chemical comparison of edible and toxic forms was done in order to highlight differences between both forms. Dichloromethane leaf extracts from toxic and edible trees were analyzed by gas chromatography. Phenolic profile and volatile compounds from fruits extracts were studied respectively by High Performance Liquid Chromatography-mass spectrometry (HPLC-MS) and Gas Chromatography Mass Spectrometry (GC-MS). Cytotoxicity effect of fruits extracts was evaluated on murine macrophage cells J774 A1. GC-analysis of dichloromethane leaf extracts revealed the presence of lupenone and lupeol only in toxic extracts. 6'-O-galloyl-epiheterodendrin and isovaleronitrile were detected in toxic pulp. However, no cytotoxic effect was found in our conditions. This study has given the opportunity to identify within the same species, compounds which could differentiate both edible and toxic forms. Nevertheless further studies are needed to better understand which compounds are responsible for toxicity in the toxic form.

Key words: Ditax, *Detarium senegalense*, toxicity, lupeol, lupenone, cyanogenic glycoside, isovaleronitrile.

INTRODUCTION

In Senegal, *Detarium senegalense* J. F. Gmel generates a great economic activity in the central and southern regions. The fruit called "ditax" in Wolof, is a globulous drupe from 3 to 8 cm in diameter, with a large central core surrounded by a farinaceous, greenish, fibrous, acidulated and sweetened pulp (Kerharo and Adam,

*Corresponding author. E-mail: ndiop@ita.sn. Tel: +221/77635 94 64.

1974; Haddad, 2000; Arbonnier, 2002). The fruits are very appreciated and widely consumed as nectar or fresh (Diop et al., 2010). However, in some areas, toxic and edible trees cohabit with a great difficulty to identify and differentiate them morphologically. By describing *D. senegalense* species, Guillemin et al. (1830), observed that this tree could produce edible or toxic fruit. Cases of intoxications have been reported one century and half ago but the compounds responsible for this toxicity have not been identified (Heckel and Schlagdenhauffen, 1889; Paris and Moyse-Mignon, 1947; D'Almeida, 1984; Imbert and Teyssier, 1986; Adam et al., 1991; Berthelot et al., 2000). Among previous studies which have been done on this field, we can mention Sambuc who did the first study on chemistry of *D. senegalense* fruits by giving an alcohol toxic fruits extract to dogs but no side effect was observed (Sambuc, 1887; Diatta, 1995; Cavin, 2007). Heckel and Schlagdenhauffen (1889) studied also toxicity of *D. senegalense*. According to them, toxicity of *D. senegalense* fruit is due to a volatile compound. In 1947, Paris and Moyse-Mignon have succeeded to isolate from toxic fruits, a bitter compound which is responsible for the toxicity according to them. They concluded that this compound appears to depress the central nervous system after transiently excited.

In Senegal, the distinction is based on the knowledge of the local population that collects only trees whose fruits are consumed by animals. Moreover, there are no objective methods to differentiate the two forms. In order to highlight difference between toxic and edible form of *D. senegalense* and to find objective parameters for their distinction, a chemical study was done by comparing their composition.

MATERIALS AND METHODS

Leaves and fruits

Leaves and fruits from edible and non-edible varieties of *D. senegalense* harvested in 2009 (from 1 adult toxic tree and 1 adult edible tree) and 2012 (from 5 different adult toxic and edible trees) in Ziguinchor were studied. Leaves were dried between two papers at room temperature and stored away from light. Fruits were frozen and stored at -18°C just after harvesting.

Gas chromatography analysis of leaf extracts

Leaves from toxic and edible old trees (1.4 g) were crushed and macerated in 10 ml of dichloromethane (Merck, Darmstadt, Germany) under agitation for 1 h in an ultrasounds water bath (88154, Bioblock Scientific - Fisher, Illkirch, France). Extracts (three replicates) were filtered, then solvent was evaporated for 10 min at 35°C with a centrivap concentrator (Labconco, Kansas City, USA). Extracts were stored for 48 h at 4°C before analysis by gas chromatography using a Trace GC Thermo Quest chromatograph / Mass detector Trace ms plus (Courtaboeuf, France). Helium was used as carrier gas at 1mL/min. Separation was carried out on a capillary column RTX 5 (30 m X 0.25 mm I.D., 0.25 μm, Restek, France) with the following temperature program: 50°C for 5 min then slope of 5°C/min up to 270°C and held for 15 min. Injector temperature was 250°C, in splitless mode. Tentative identification of peaks was done by comparing mass spectra with those of the National Institute of Standards and Technology (NIST) data base. Standards of lupenone and lupeol (Sigma Aldrich & Fluka, Saint-Quentin Fallavier, France) were injected each one separately, then after mixing together. Toxic and edible leaf extracts with and without lupenone and lupeol standards were also analyzed by GC-MS.

Phenolic profile of fruit pulp

Edible fruit pulp was extracted from cores and fiber network surrounding the core. Toxic fruit pulp was extracted at the same way. Phenolic compounds were extracted from each toxic and edible pulp fruit by stirring for 10 min, 500 mg of pulp in 20 ml of acetone. After filtration and evaporation, extract was diluted in 2 ml of a mixture of methanol – water 50/50 then filtered at 0.45 µm. Pulp extracts were then analyzed by High Performance Liquid Chromatography – Mass Spectroscopy (HPLC-MS) using an HPLC SURVEYOR, equipped with a DAD detector UV 6000 LP and coupled with a mass spectrometer LCQ (THERMO FINNIGAN, San José, USA). Separation was done on an ACE C-18 column (250 mm X 4.6 mm, 5 µm, France HAS) thermostated at 30°C in reversed phase. Mobile phases were water / formic acid (0.1%) for eluant A and water / acetonitrile / formic acid (19.9/80/0.1) for eluant B. The following binary gradient was used with a flow of 0.7 $mL.min^{-1}$: T = 0 min : 5% B ; T = 50 min : 35% B ; T = 55 min : 50% B; T = 60 min : 80% B ; T = 65 min : 100% B ; T = 70 min : 100% B ; T = 72 min : 5% B ; T = 85 min : 5% B. Volume injection was 10 µl. Detection was carried out at 280, 330 and 360 nm. The electro-nebulization was carried out in negative mode.

Volatile profile of fruits by solid phase micro extraction (SPME) and gas chromatography (GC) / mass spectrometry (MS) analysis

Eight edible whole fruits and 8 toxic whole fruits were set in glass jars with a septum in the top. Micro extraction was carried out for 2 h at ambient temperature using a polydimethylsiloxane-divynilbenzene 65 µm fiber (StableFlex ^{TM} SPME fiber, Supelco). A tandem gas chromatograph 6890 / MSD 5973 / Gerstel Multipurpose Sample MPS-2 was used for volatiles compounds analysis (Agilent Technologies, Palo Alto, USA). After trapping, injection was carried out in splitless mode at 250°C for 180 s for desorption. Separation was done on a DB-WAX polar column (J&W, 30 m x 0.25 mm x 0.25 µm). The following temperature program was used: 40°C for 1 min; 3°C/min from 40°C to 170°C, then 10°C/min up to 240°C and held for 10 min. Mass spectra were recorded in Electron Ionization impact (EI+) mode at 70 eV within 40 to 300 Da. Source temperature was 250°C and helium was used as carrier gas at 1 mL/min. Isovaleronitrile standard was used as internal standard for identification and quantification in toxic pulp extract.

Cellular toxicity evaluation of fruit extracts by MTS/PMS assay

The cellular toxicity of pulp and husk from edible and toxic fruits was evaluated using a J774 A1 cells of murine macrophages obtained from the American Type Culture Collection (ATCC, TIB67, Rockville, MD) by the technique of MTS/PMS as previously described by Dussossoy et al. (2011).

Preparation of fruits extracts

Methanol extracts were prepared from toxic and edible fruits.

Briefly, 10 g of toxic pulp and 10 g of toxic husk were stirred 10 min with 5 ml of water and 40 ml of methanol. The liquid phase was filtered on paper and the residue was extracted again with 20 ml of methanol for 10 min. The methanol phase was evaporated under vacuum and the crude extract was taken up in 2 ml of methanol. Ten ml of diethyl ether were added to remove chlorophyll pigments. For edible extracts, 5 g of pulp and 5 g of husk were used.

After that, 40 ml of phosphate saline buffered (PBS) were added to each methanol extracts, which were filtered through 0.45 µm and diluted with the culture medium at the concentration of 20 mg/ml. The following concentrations (mg/ml) were tested on the cells: 10 – 5 – 1 - 0.5 - 0.1 - 0.05 -0.01 - 0.005 - 0.001 - 0.0005 and 0.0001. Each concentration was repeated 6 times. The toxicity of isovaleronitrile standard was also evaluated on J774 A1 cells at 1-0.5 –0.1 – 0.05 – 0.01 – 0.05 – 0.001 – 0.0005%.

Cells preparation and cytotoxicity evaluation

Briefly, 1 x 10^5 cells/well were seeded in a 96-well plate in RPMI (*Roswell Park Memorial Institute medium* 1640 with Glutamax® supplemented with 100 µg/ml of streptomycin, 100 Units/ml of penicillin and 10% heat inactivated fetal bovine serum, Gibco Life Technologies, Grand Island, NY, USA) and incubated with various concentrations of extracts under 200 µl for 20 h at 37°C in a humidified incubator containing 5% CO_2. After incubation, 20 µl/well of a tetrazolium salt MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, Promega Corporation, 2007), mixed with an electron-coupling reagent (PMS, phenazine methosulfate) diluted in PBS, was added. The plate was incubated for another 4 h at 37°C. Dehydrogenase enzymes found in metabolically active cells accomplish the conversion of MTS into soluble formazan. The quantity of formazan product was measured by the amount of the absorbance at 490 nm, which is directly proportional to the living cells in culture.

RESULTS AND DISCUSSION

Gas chromatography analysis of leaf extracts

Dichloromethane leaf extracts from edible and toxic adult trees were analyzed by gas chromatography in order to highlight presence or absence of at least one compound, which could be used as criterion of differentiation between the two forms. Mass spectra of some unidentified compounds eluted between 55 and 60 min, let think that these compounds could belong to the triterpenes family like lupeol and lupenone. Lupenone and lupeol standards were analyzed each one separately and then together (Figure 1). Mass spectra of lupenone and lupeol were shown in Figure 2. Leaf extracts of edible and toxic trees were then analyzed by GC-MS, without standards and with standards. Retention time of lupenone and lupeol were 56.5 and 57.0 min respectively with interval time ranging between 56 and 58 min (Figure 3). Lupenone and lupeol were detected only in toxic leaf extracts (Figure 3c and d). It is the first time that lupenone and lupeol were found in *D. senegalense*. Lupeol is a triterpenic alcohol present in various families of plants. It is one of the most active compounds of several medicinal plants. Lupeol is found in grape, hazel nut, olive oil, cocoa butter, cabbage (Gallo and Sarachine, 2009), mango pulp (Duke, 1992) and tamarind (Imam et al., 2007). Lupeol has many pharmacological activities in particular anti-protozooairy (Fotie et al., 2006; Hoet et al., 2007; Ajaiyeoba et al., 2008), anti-inflammatory (Theophile et al., 2006; Rocha et al., 2008; Vasconcelos et al., 2008) and antitumor (Saleem et al., 2008; Cmoch et al., 2008; Prasad et al., 2008; Saleem, 2009). Even if toxicity of lupeol is not well established (Imam et al., 2007; Fotie et al., 2006; Hoet et al., 2007), it could be used to identify toxic and edible forms because they are present only in toxic leaves. Nevertheless, their presence or absence in toxic and edible fruits has to be done in perspective.

Phenolic profile of fruit pulp

Profiles of phenolic compounds from edible and toxic fruit pulps were compared on the basis of UV-VISIBLE spectrum, mass of molecular ions and ions fragments as well as using data from the literature. Figure 4 shows chromatograms of pulp extract from edible and toxic fruit recorded at 280 nm. Table 1 presents phenolic compounds identified in edible and toxic fruit pulps of *D. senegalense*. The identified compounds are primarily flavanols, dimers of catechine and epicatechine as well as derivatives of catechine and catechine gallate. Galloyl derivatives of shikimic acid were also observed. The gallic acid was also present. This result agrees with those of Haddad (2000) who found 7.05 mg/kg of gallic acid in the edible pulp of ditax. The main difference between phenolic profiles of the two chromatograms was the presence of a phenolic compound eluted at 36.74 min only in the toxic fruit pulp extract. This compound, characterized by a λ_{max} of 274 nm and a m/z of 412.11 is very close to the 6'-O-galloyl-(R)-epiheterodendrin (λ_{max} 275 nm; m/z 412) found by Cavin (2007). According to this author, this compound is formed by a β-glucose linked with isovaleronitile and a gallic acid in esterified form (Figure 5). When isovaleronitrile is linked in 1' by a β-glucose, the molecule is named heterodendrin or epiheterodendrin, according to the absolute configuration S or R, respectively of C-2 (Jaroszewski, 1986; Lechtenberg et al., 1996; Nielsen et al., 2002). Our results seem to confirm the implication of 6'-O-galloyl-(R)-epiheterodendrin in the toxicity of *D. senegalense* fruit as previously noticed by Cavin (2007). Indeed, this compound seems to belong to the class of cyanogenic glycosides. Cyanogenesis is the ability of some plants to synthesize cyanogenic glycosides, which when enzymatically hydrolyzed, release cyanohydric acid (HCN) (Francisco and Pinotti, 2000; Harborne, 1972). ß-glucosidase enzyme is responsible in most cases, for the hydrolysis reaction, which produces sugars and a cyanohydrin that spontaneously decomposes to HCN and a ketone or an aldehyde (Figure 6). Hydroxynitrile lyase enzyme can also catalyze the HCN release reaction from cyanohydrin (Harborne, 1993). However, according to

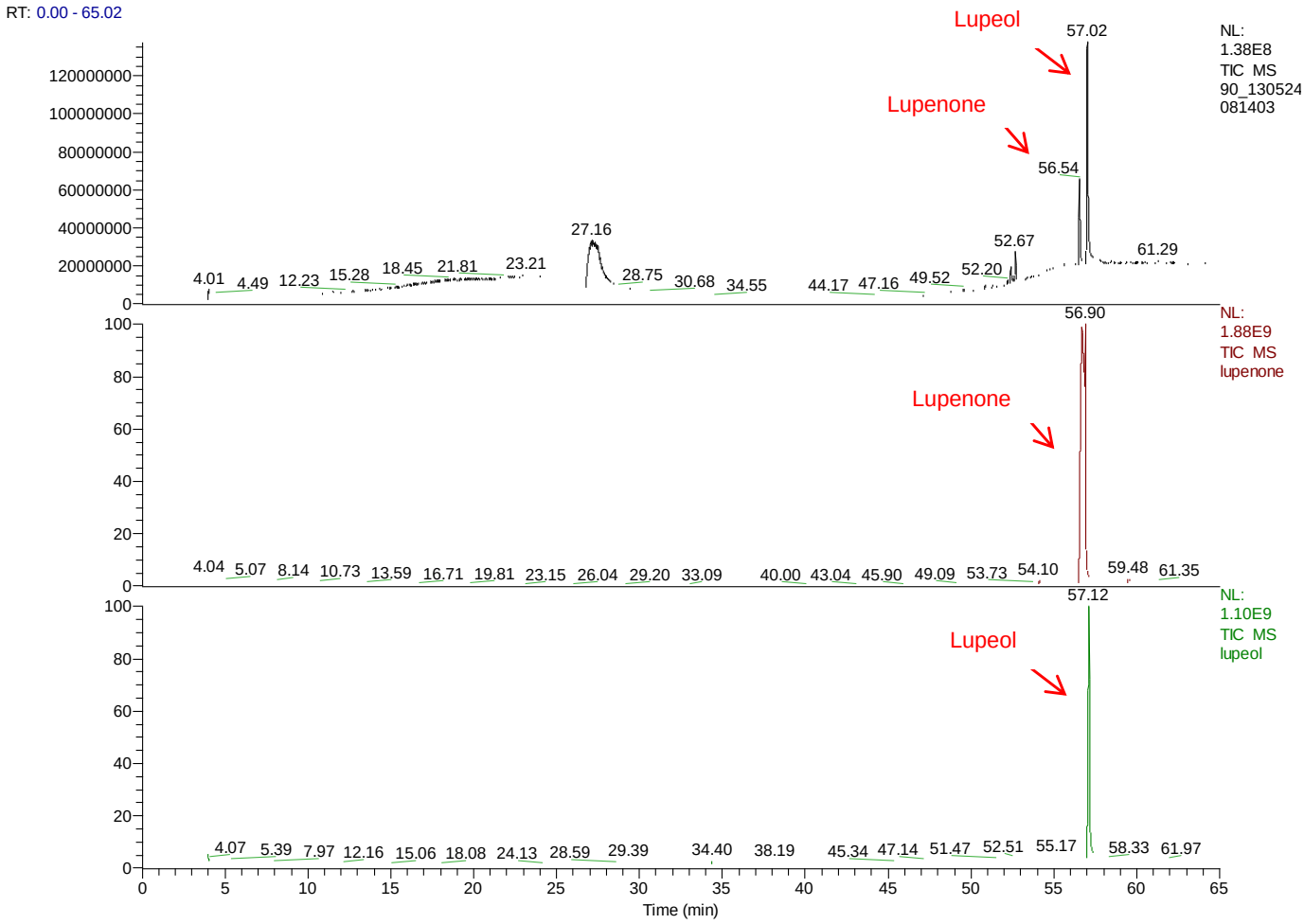

Figure 1. Chromatograms of standards: (a) lupenone and lupeol together; (b) lupenone alone; (c) lupeol alone.

Francisco and Pinotti (2000), HCN of cyanogenic glycoside is not release enzymatically in some cases like in *Rapanea umbellata* plant. Cyanogenic glycosides are formed from amino acids, and are classified according to the nature of them (Cavin, 2007). According to Lechtenberg et al. (1996), isovaleronitrile linked to glucose of 6'-O-galloyl epiheterodendrin probably comes from to L-leucine as presented in Figure 7.

The description of a bitter almond odor, which would be released from the toxic fruits related by Berthelot et al. (2000), seems to confirm the involvement of 6'-O-galloyl-(R)-epiheterodendrin because cyanogenic glycosides have a bitter almond odor (Paris and Moyse-Mignon, 1947; D'Almeida, 1984). Moreover, the release of HCN was confirmed by Cavin (2007) by Prussian blue formation.

Volatile profile of fruits

Since animals, in particular monkeys, do not consume toxic fruits because of their bitter odor (Berthelot et al., 2000), volatile compounds from whole fruits were extracted by solid phase micro-extraction and analyzed by GC-MS to seek an eventual difference between toxic and edible fruits. Figure 8 shows volatile profiles of the two extracts and the principal difference between the two profiles is the detection of isovaleronitrile at 7.12 min retention time only in the whole toxic fruits. Presence of isovaleronitrile in toxic fruit pulp could explain the bitter almond odor release from toxic fruits and related by some authors (Berthelot et al., 2000; Adam et al., 1991). Chromatogram of whole fruits extracts between 6 and 7.3 min as well as the mass spectrum of isovaleronitrile are presented in Figure 8b. Thereafter, isovaleronitrile was sought in toxic and edible pulps with a standard of isovaleronitrile. Isovaleronitrile was detected only in toxic pulp extracts at 37.3 nmol/mg per fresh fruit pulp.

Detection of isovaleronitrile only in toxic fruit pulp seems to confirm presence of 6'-O-galloyl-(R)-epiheterodendrin in toxic fruits. Moreover, according to Heckel and Schlagdenhauffen (1889), it is a volatile

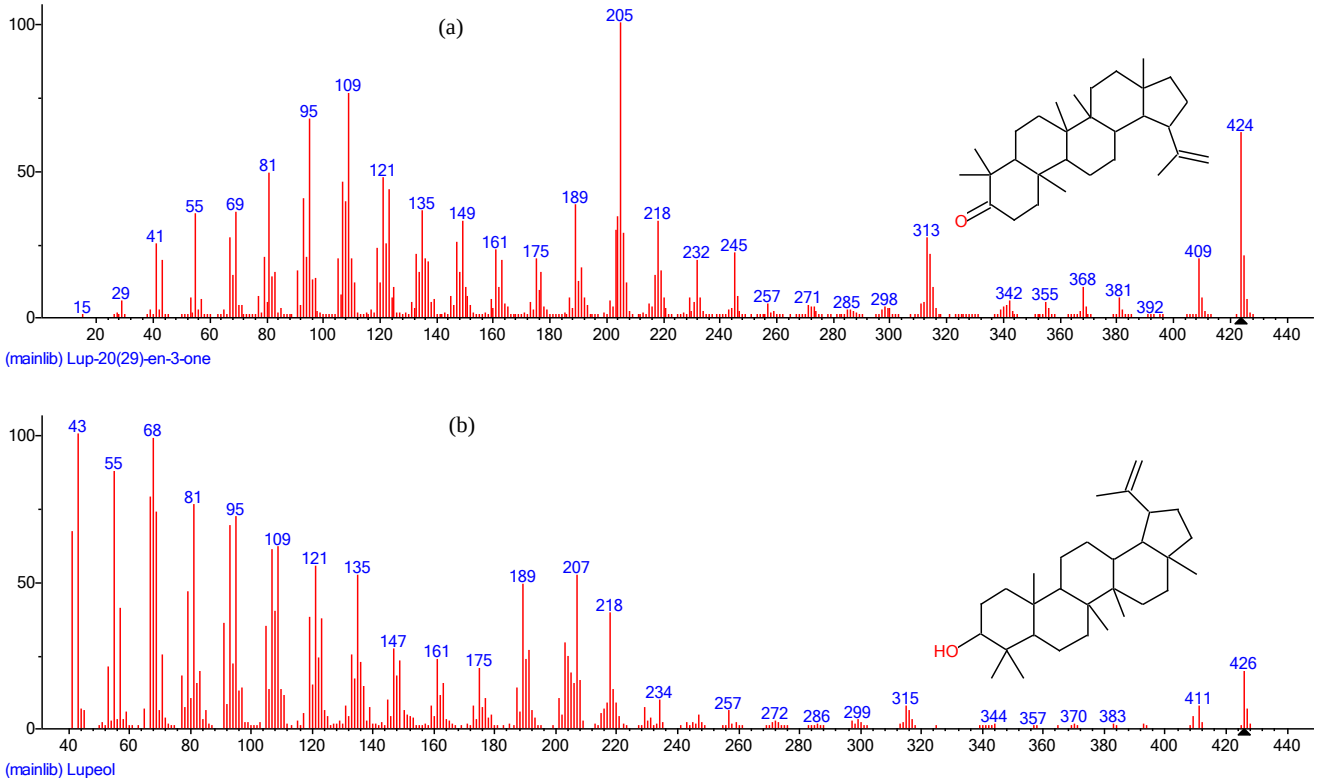

Figure 2. Mass spectra of standards: (a) lupenone; (b) lupeol.

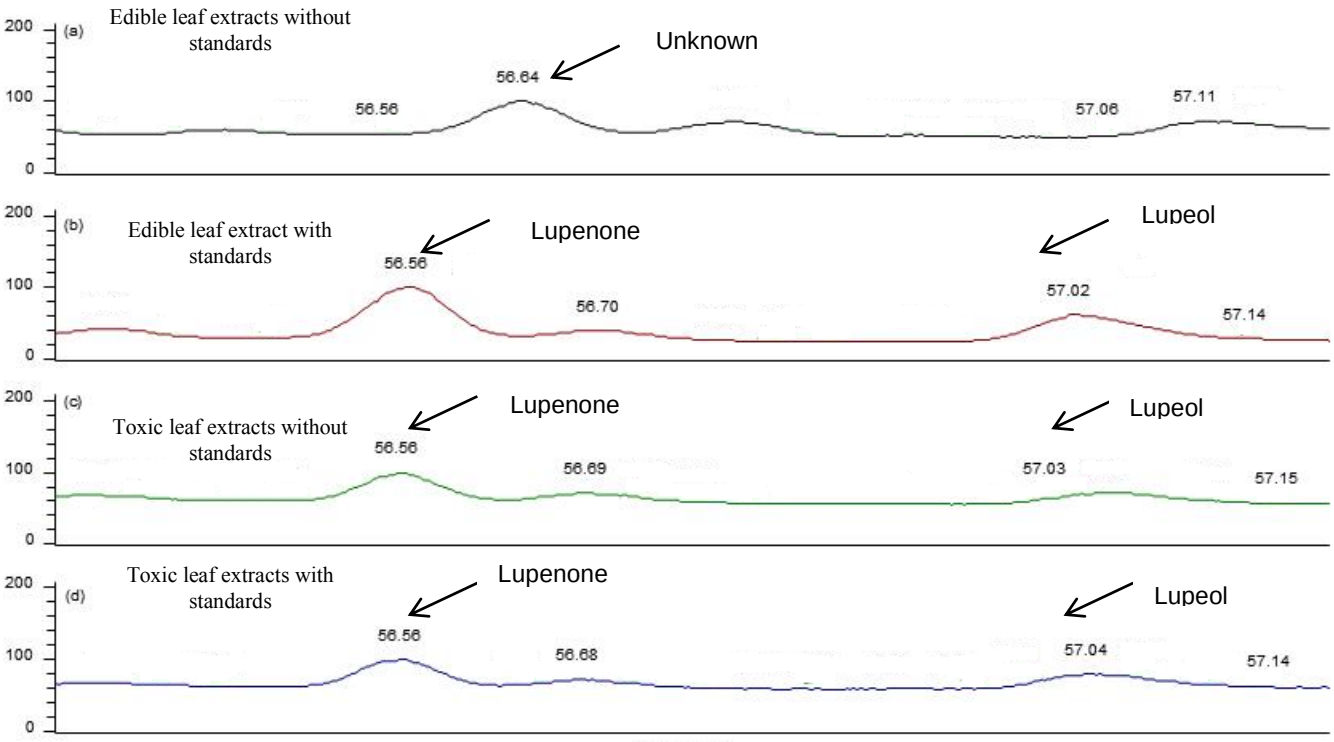

Figure 3. Chromatograms of dichloromethane leaf extracts from *Detarium senegalense* between retention times 56 to 58 min: (a) dichloromethane extract of edible leaves; (b) dichloromethane extract of edible leaves with lupenone and lupeol add as standards; (c) dichloromethane extract of toxic leaves; (d) dichloromethane extract of toxic leaves with lupenone and lupeol add as standards.

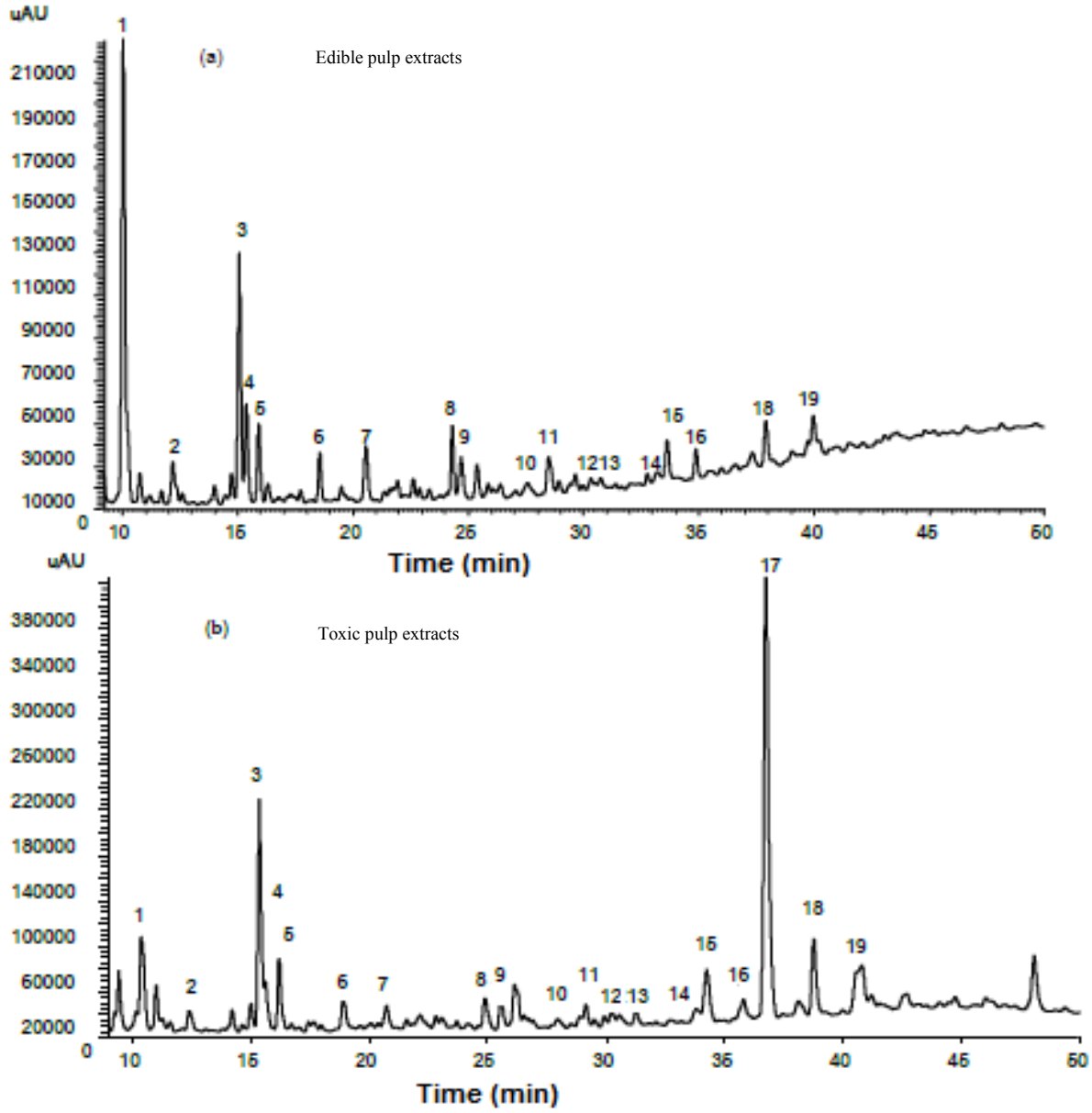

Figure 4. Chromatogram HPLC of phenolic compounds (detection at 280 nm) of edible (a) and toxic (b) pulp extracts of *Detarium senegalense*. (cf. Table 1 for peaks identification).

compound that would be responsible for toxicity.

Evaluation of cellular toxicity

Our study revealed that both toxic and edible fruit extracts had no toxic effect on viability of murine cells J774 (Figure 9). In the same way, isovaleronitrile did not have toxic effect on cell viability even if a slight decrease of absorbance was noticed (from 1.27 to 1.13) between 0.1 and 1% (Figure 10). These extracts have now to be tested on other cellular models especially on human cells to evaluate their potential toxicity in human. Nevertheless, this test is an in vitro test, which gives an indication of cytotoxicity but in any case not presage an in vivo toxicity in humans after ingestion.

Conclusion

Our results highlighted differences between the edible and toxic forms of *D. senegalense*. Analysis of phenolic compounds revealed the presence, only in the toxic form, of a cyanogen glycoside: the 6'-O-galloyl epiheterodendrin.

Table 1. Phenolic compounds identified in edible and toxic pulp of *D. senegalense* fruits.

Pics	Tr (min)	UV	MS	MS^2	Tentative of identification
1	10-10.3	271	169		Gallic acid
2	12.2	273	493		n.i.
3	15.1-15.3	275	325	169	Galloyl of shikimic acid
4	15.4-15.6	274	325	169	Galloyl of shikimic acid
5	15.9-16.2	275	325	169	Galloyl of shikimic acid
6	18.6-18.9	273	487	443,407, 271	n.i.
7	20.6-20.7	278. 290sh	519	473,451, 443	n.i.
8	24.3-24.6	270	483	331,271, 169	Digalloyl glucose
9	24.7-24.8	278	289	245, 205, 179	Catechine (cat)
10	27.6-27.9	278	577	509,425, 407	Procyanidine dimere
11	28.5-29.1	256sh. 278	481	437, 313,169	n.i.
12	30.3-30.5	278	289	245, 205, 179	Epicatechine (épicat)
13	30.8-31.2	274	729	577, 407, 289	Cat-cat gallate
14	32.8-33.2	274	635	591, 483, 331	Trigalloyl glucose
15	33.6-34	278	729	577, 407, 289	Epicat-Cat-gallate
16	34.9-35.6	272	609	565, 457, 271	n..i
17	***36.7***	***274***	***412***	***313, 169***	***6'-O-galloyl-(R)- epiheterodendrin***
18	37.9-38.7	278	881	729, 577,289	Cat-gallate/cat-gallate
19	40-40.7	278	441	289	Cat-gallate

n.i. : unidentified.

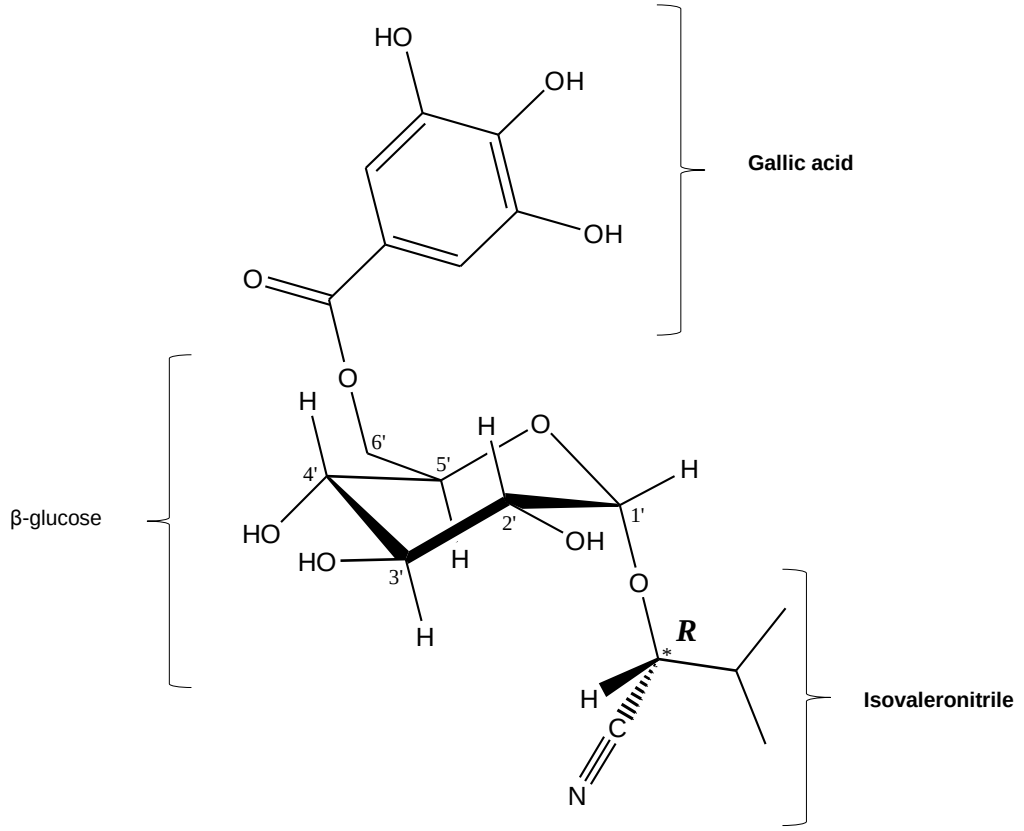

Figure 5. Chemical structure of 6'-O-galloyl-(R)-epiheterodendrin isolated from toxic fruit pulp of *D. senegalense* by Cavin (2007).

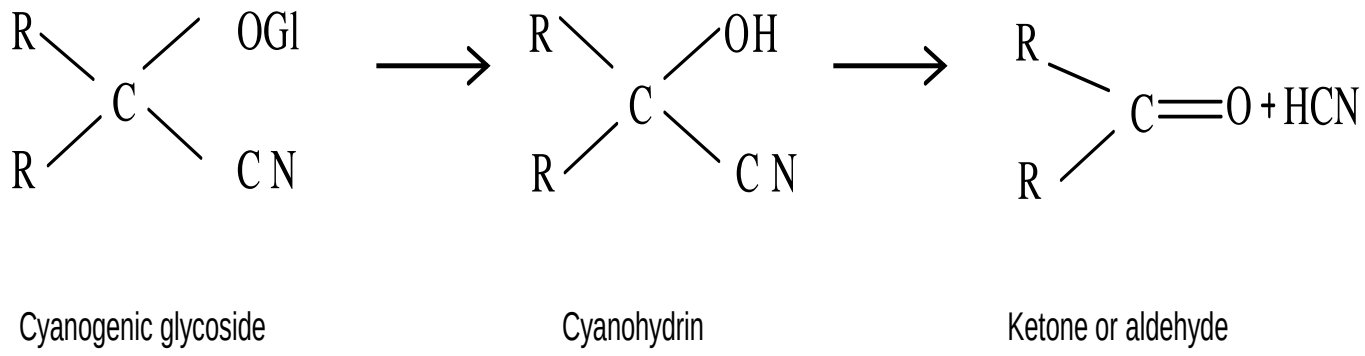

Figure 6. Pathway of HCN by cyanogenic glycoside plants (Francesco and Pinotti, 2000).

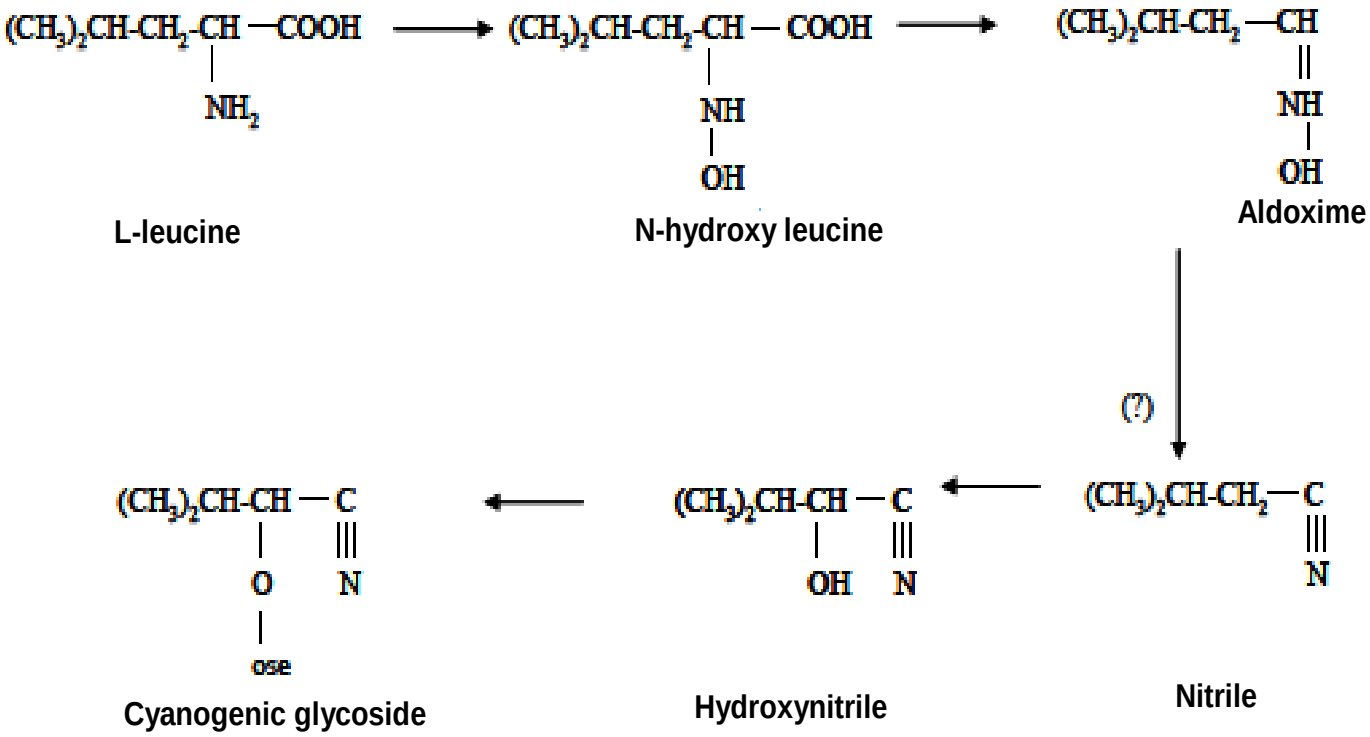

Figure 7. Biogenetic pathway of 6'-O-galloyl-(R)-epiheterodendrin formation (Cavin, 2007; Lechtenberg et al. 1996; Bruneton, 1999).

Lupeol and lupenone were also detected only in the toxic leaf extracts. Quantification of 6'-O-galloyl epiheterodendrin, lupeol and lupenone in fruits and leaves could be used to distinguish the edible and toxic forms. The development of a rapid method to detect isovaleronitrile, lupeol or lupenone could be considered in order to quickly classify fruits. Nevertheless, it would be interesting to enlarge tree sampling with different geographical origins and to validate reproducibility of the results. Complementary work would be carried out in order to identify the compounds responsible for the toxicity.

Conflict of Interests

The authors have not declared any conflict of interests.

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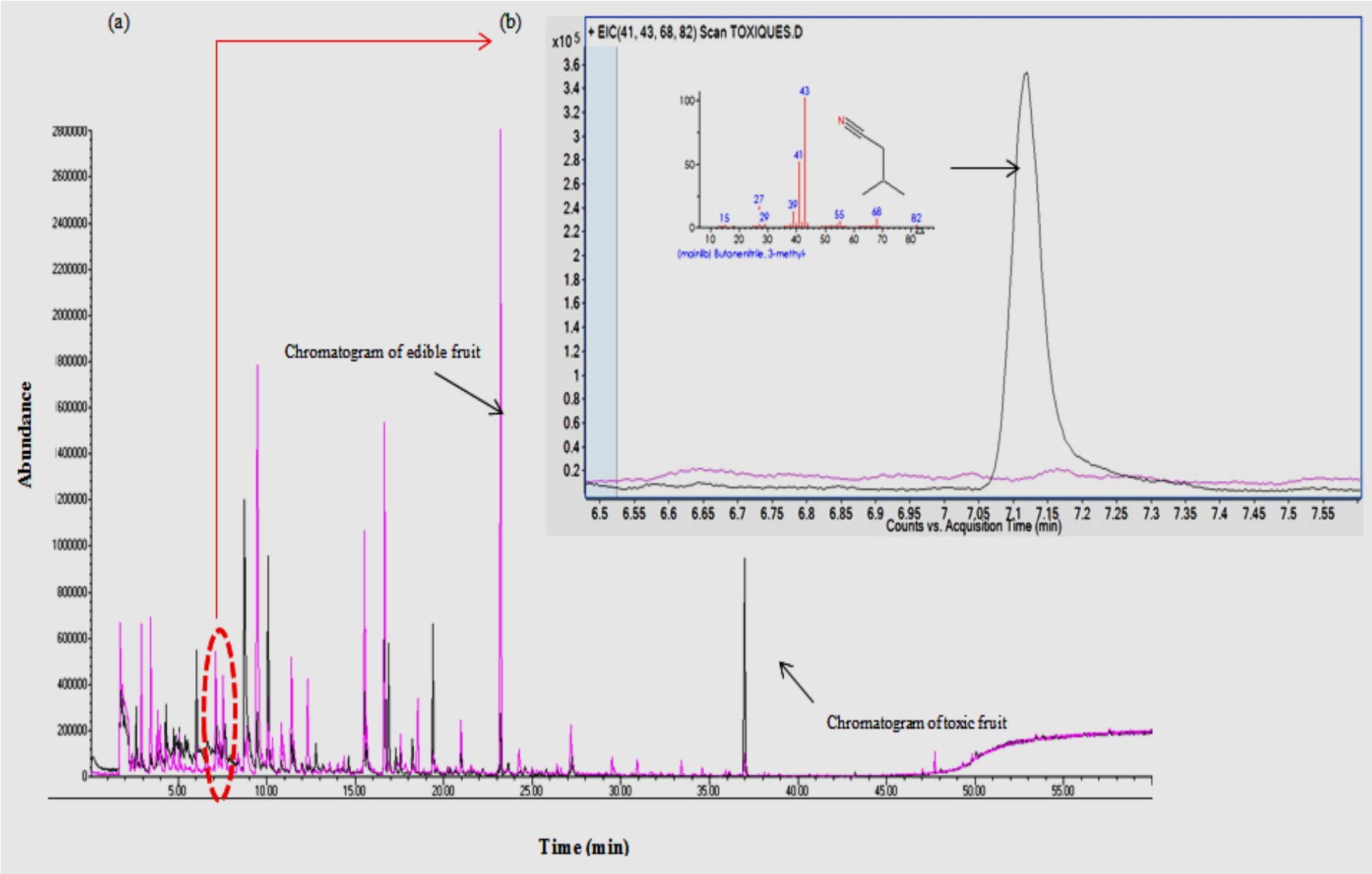

Figure 8. Comparison of volatiles compounds (a) extracted by solid phase micro extraction (SPME) on whole toxic fruits (black line) and whole edible fruits (pink line) of *D. senegalense* ; (b) zoomed chromatograms between 6.5 and 7.5 min with the characteristic ions of isovaleronitrile (41,43,68,42).

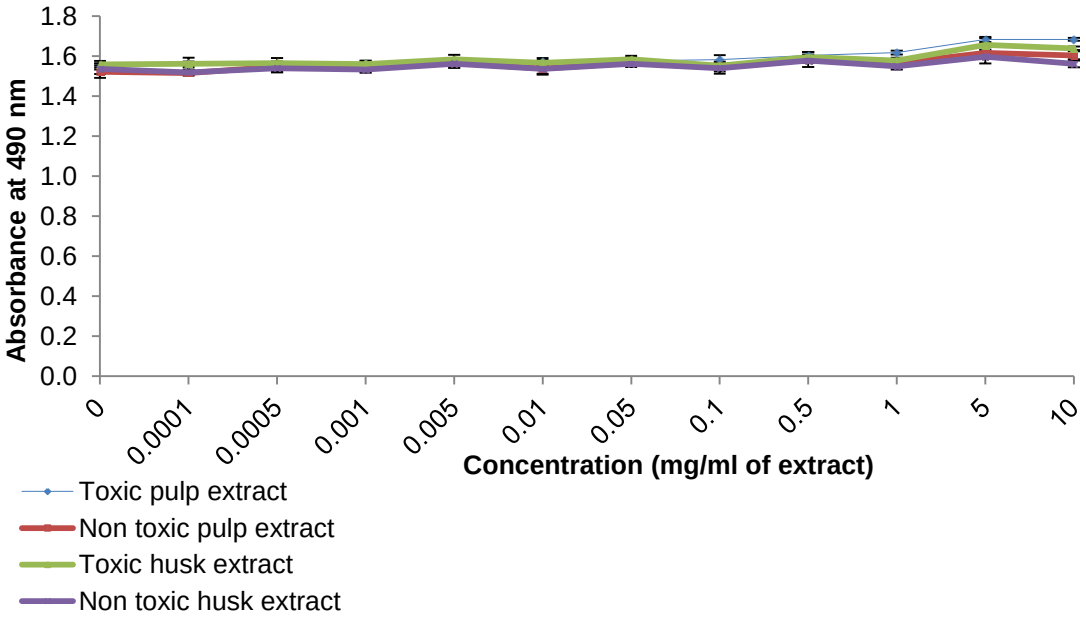

Figure 9. Evaluation of cellular toxicity of toxic and non-toxic methanolic pulp and husk extracts from *D. senegalense* on J774 A1 cells of murine macrophages after 6 h treatment (means and standard deviation for 6 assays).

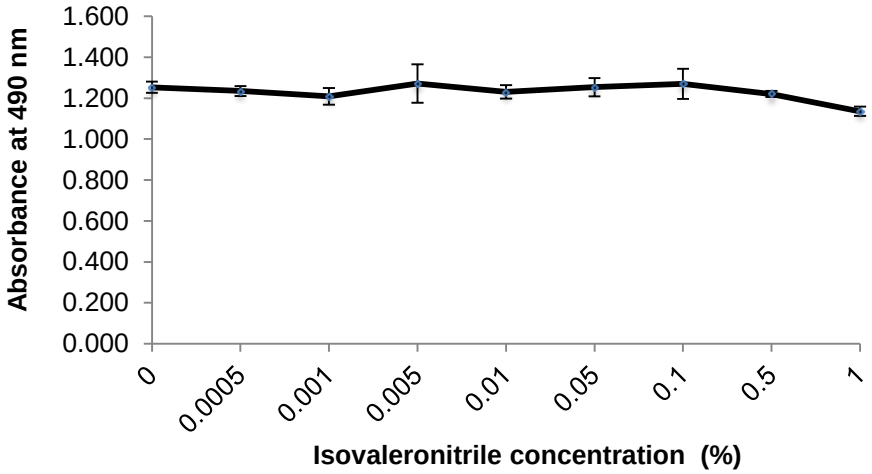

Figure 10. Evaluation of cellular toxicity of isovaleronitrile pure standard on J774 A1 cells of murine macrophages after 6 h treatment (means and standard deviation for 6 assays).

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