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Pharmacological Studies of Ten Medicinal Plants Used for Analgesic Purposes in Congo Brazzaville

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Abstract: The aim of this study was to assess ten plants from the Congolese pharmacopoeia for their analgesic activity as well as their cytotoxicity, in order to validate their traditional use. Thirty-three extracts were obtained in 3 different solvents (Water, Ethanol, DCM) from these ten plants selected after an ethnobotanic survey in the region of the Pool (Congo Brazzaville): *Leonotis nepetaefolia* (Lamiaceae), *Manotes pruinosa* (Connaraceae), *Spilanthes uliginosa* (Asteraceae), *Hymenocardia ulmoides* (Euphorbiaceae), *Celosia trigyna* (Amaranthaceae), *Cogniauxia podolaena* (Cucurbitaceae), *Brillantaisia patula* (Acanthaceae), *Urena lobata* (Malvaceae) , *Mitracarpus scaber* (Rubiaceae), *Triumfetta rhomboidea* (Tiliaceae). The writhing test (Siegmund Chemical Test) was used for the pharmacological screening. The cytotoxicity of all the extracts was tested on KB (Human epidermoid carcinoma) and Vero (African green monkey kidney) cell lines with taxotere as positive control. A TLC chemical screening of the extracts was carried out to detect the major chemical classes present in the plants. The data of the traditional medicine were confirmed, since eight plants out of ten were active, aqueous and ethanolic extracts being the most active. Moreover, only *C. podolaena* leaf extracts were cytotoxic (87% of inhibition on KB). This work opens the way for the research of the active molecules from these plants and for their use as leads in the synthesis and the pharmacomodulation of compounds with analgesic potentiality.

Key words: Analgesic, anti-nociceptive, medicinal plants, Congo pharmacopoeia, cytotoxicity

INTRODUCTION

The African pharmacopoeia is rich in plants having a wide range of pharmacological properties, resulting from the various chemical compounds that they contain. Some of them are currently in clinical trial to develop new plant drugs, such as *Physostigma venenosum* for Alzheimer disease or *Coix lachryma-jobi* for cancer (Saklani and Kutty, 2008). Concerning the treatment of pain, the relevant literature indicates that more than 300 plants growing in Africa have been assayed and proven active in vivo. However, a systematic study is still wanting, even if this work has been well begun by South Africa (Ojewole, 2005, 2006, 2007, 2008) and Nigeria (Nwafor and Okwuasaba, 2003; Omisore *et al*., 2004; Mbagwu *et al*., 2007). Two of these 300 plants, *Sutherlandia frutescens* (L). R.Br. and *Harpagophytum procumbens* DC. are powerful anti-inflammatory drugs (Ojewole, 2004; Mahomed and Ojewole, 2004) and a relation between their activity and the prevention of cancer has been described (Kundu *et al*., 2005). These plants, well known and widely marketed in the world, are thus promising to develop future African plant drugs. Available biological data for Congo show that few studies concern potentially

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analgesic plants, despite the widespread traditional use of plants in pain treatment (Banzouzi *et al*., 2008a; Okiemy-Andissa *et al*., 2004). Medecins d'Afrique's surveys as well as the recommendations of tradipractitioners incited the authors of this article to begin a systematic inventory of Congolese plants used for the treatment of pain. This allowed them to list 200 plants among which 51 were published (Banzouzi *et al*., 2008a). In this study ten of these plants were assessed for their analgesic activity as well as their cytotoxicity, in order to validate their traditional use. These plants were chosen both for their availability and because they were not yet studied in laboratory. Indeed five of them had never been studied at all. The principal chemical classes in the plants were detected thanks to a Thin Layer Chromatrography (TLC) screening.

MATERIALS AND METHODS

Plant material: The 10 plants were collected in December 2006, in the region of the Pool (Congo Brazzaville) and dried before being crushed and sent to ICSN-CNRS in France. The botanical identification of each plant was confirmed by the botanists of CERVE and a voucher specimen was deposited at their Herbarium. Reference numbers are given in Table 1.

Preparation of the extracts: For each of the 10 plants, 3 extracts were obtained respectively with DCM, ethanol and water. The plant material was first extracted in DCM then the residual marc was extracted in ethanol. To be in accordance with the traditional mode of use, aqueous extracts for each of the plants were also prepared. After filtration and centrifugation (3000 rpm for 20 min, C3-select centrifuge, Biotech) the aqueous extracts were freeze-dried (LL300 freeze dryer, Thermo scientific) to obtain dry extracts. Leave and bark of *H. ulmoides* were extracted separately, since they are never used together in traditional remedies. Thirty-three extracts were therefore obtained.

Chemical TLC screening: The extracts were screened for the presence of tannins, saponins, terpenoids, iridoids, alkaloids, anthraquinones, flavonoids, lactones/esters and protein/amino acids on TLC silicagel 60 F_{254} plates (Merck). Development was carried out with the following solvent system: chloroform/ethyl acetate/methanol/water (17:47:26:10). The positive controls used were: cinchonidine (alkaloids), quercetin (flavonoids), λ-valerolactone (lactones/ esters), L-Trp, L-Phe (amino-acids, proteins), lupeol (terpenoids), catalpol (iridoids), 9,10-anthraquinone (anthraquinones), saponarioside C (saponins), ellagic and gallic acids (tannins), glucose (carbohydrates). After development, the plates were sprayed with the appropriate reagents: Dragendorff reagent (alkaloids), Neu reagent (flavonoids), hydroxylamine-ferric chloride (lactones/esters), ninhydrine (protein/amino acids), Liebermann reagent (terpenoids), vanilline-sulphuric acid (iridoids), KOH (anthraquinones), anisaldehyde-sulphuric acid (saponins), ferric chloride (tannins). Reagents were prepared according to Stahl (1969). Detection was carried out visually in visible light and under UV light (λ = 254 nm or 365 nm).

Cytotoxicity: The cytotoxicity of the extracts was assayed against KB (Human epidermoid carcinoma) and Vero (African green monkey kidney) cells (ICSN, France) grown in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 25 mM glucose 10% (v/v) fetal calf serum, 100 UI penicillin, 100 μg mL^{-1} streptomycin and 1.5 μg mL^{-1} fungizone and kept under 5% CO_2 at 37°C. Ninety-six-well plates were seeded with 600 KB cells per well in 200 μL medium. Twenty-four hours later, plant extracts dissolved in dimethyl Sulfoxide (DMSO) at a stock solution of 10 mg mL^{-1} were added for 72 h

Table 1: Botanical, chemical and extraction data

Species	Family	Voucher number	Code	Plant parts	Solvents	Yield (%)	Chemical classes
Leonotis nepetaefolia (L.) W.T.Aiton	Lamiaceae	MK028	LN	whole plant	DCM Ethanol water	1,4 7.1 6.1	LE, TE, IR, SA
Manotes pruinosa Gilg	Connaraceae	MK052	MP	leaves	DCM Ethano water	1 2,81.4	FL, LE, AA, IR, AQ, SA,TA
Spilanthes uliginosa Sw	Asteraceae	MK012	SU	whole plant	DCM Ethanol water	2,33.812.3	FL, LE, TE, AQ, SA
Hymenocardia ulmoides	Euphorbiaceae	MK021	HUB	bark	DCM Ethanol water	0,86.37.2	LE, TE, IR, SA, TA
Hymenocardia ulmoides Oliv	Euphorbiaceae	MK021	HUL	leaves	DCM Ethanol water	3,04.416.3	FL, LE, AA, TE, IR, AQ, SA, TA
Celosia trigyna L.	Amaranthaceae	MK003	CT	leaves	DCM Ethanol water	2,310.415.4	FL, AA, TE, SA, TA
Cogniauxia podolaena Baill.	Cucurbitaceae	MK019	CP	whole plant	DCM Ethanol water	3,21.09.2	FL, LE, AA, TE, AQ, SA
Brillantaisia patula T.Anders.	Acanthaceae	MK001	BP	leaves	DCM Ethanol water	5,02.37.8	LE, TE, IR, AQ, SA
Urena lobata L.	Malvaceae	MK053	UL	leaves	DCM Ethanol water	2,50.38.7	FL, LE SA
Mitracarpus scaber Zucc.	Rubiaceae	MK054	MS	whole plant	DCM Ethanol water	3,41.419.0	FL, LE, TE, IR, SA, TA, SA
Triumfetta rhomboidea Jacq.	Tiliaceae	MK049	TR	leaves	DCM Ethanol water	3,21.38.6	FL, LE, TE, IR, AQ, SA

AL: Alkaloids ; FL: Flavonoids; LE: Lactones/Esters;AL: Alkaloids ; FL: Flavonoids; LE: Lactones/Esters; AA: Proteins/Amino acids; TE: Terpenoids; IR: Iridoids; AQ: Anthraquinones; SA: Saponins; TA: Tannins Proteins/Amino acids; TE: Terpenoids; IR: Iridoids; AQ: Anthraquinones; SA: Saponins; TA: Tannins

at a final concentration of 1% in a fixed volume of DMSO. Controls received an equal volume of DMSO. After 2 h of incubation with the Methanethiosulfonate (MTS) reagent (Promega, Madison, WI) the number of viable cells was determined by measuring the optical density of every well at 490 nm with a spectrophotometer. The reference used was taxotere. The tests were performed in triplicate.

Animals for the writhing test: The animal used were male CD1 mice (Charles River, France) weighing from 18 to 20 g. Ten mice were placed randomly by cage, at 21°C with a 12h/12h night/day cycle. They were fed and watered ad libitum. The ethical rules published by International Association for the Study of Pain (Zimmerman, 1983) were respected.

Posology: The initial posology was determined from the data of the relevant literature and from the recommendations of the tradipractitioners. According to the authors, it can vary between 1g of extract by kg of weight (Diallo and Diouf, 2000) to 0.5 g kg^{-1} (Witaicenis *et al*., 2007) and come down up to 0.02 g kg^{-1} (Vongtau *et al*., 2004). Traditionally, for an adult (70-75 kg^{-1}) the Congolese tradipractitioners use a decoction of a handle of plant material, approximately 20 g, in 200-250 mL of water, administered by oral route. It was thus decided for this study to work with a dose of 0.25 g kg^{-1}, the extracts being dissolved in the DMSO 30% and administered per os. The solutions were prepared just before the tests.

Writhing test: The animals were pre-treated with the plant extracts (250 mg kg^{-1}, per os) or DMSO 30% (control group, p.o.) before the 0.6% acetic acid administration (0.01 mL g, i.p.) 30 min later (Siegmund *et al*., 1957). The animals were afterwards observed continuously for 25 min and the number of writhes (contractions of the abdominal musculature) was counted. The experiments were always run in parallel for control and treated groups and 8 to 10 animals were used for each treatment. Acetic salicylic acid (Bayer, Germany) was used as positive control, at a dose of 250 mg kg^{-1}.

Statistical analysis: Values were expressed as Mean±SEM. Statistical significance for analgesic activity was calculated using a one-way analysis of variance (ANOVA) followed by Dunnett's test (XLStat, Addinsoft). Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Extraction yield and phytochemical screening: Extraction yields and results of the chemical screening for each plant are given in Table 1. The aqueous extracts gave the higher extraction yields. The chemical screening permitted to detect flavonoids, lactones/esters, protein/amino acids, terpenoids, iridoids, anthraquinones, saponins and tannins, but there were no alkaloids.

Cytotoxicity: The percentages of inhibition of the 33 extracts measured at 10 µg mL^{-1} on KB and Vero cell lines are presented in the Table 2. In first analysis, none of the extracts was cytotoxic on Vero cells. This absence of cytotoxicity is promising for the future use of the extracts (no toxicity for kidney). Three plants, *L. nepetaefolia*, *S. uliginosa* and *T. rhomboidea* presented some cytotoxicity against KB cells. *C. podolaena* was the only really cytotoxic plant (87% of inhibition of KB). These results show that the traditional aqueous or alcoholic extracts are likely not cytotoxic. To conclude definitely on the innocuity of the active extracts, tests of acute and chronic toxicity will be necessary. The hematological and the biochemical parameters of the mice will have to be checked during the treatment.

Acetic-acid induced writhing test: The analgesic activity of the 33 plant extracts tested on mice (0.250 mg $kg,^{-1}$ per-os) comparatively to controls are given in Fig. 1-3.

None of the DCM extracts permitted a significant decrease of the number of abdominal contractions, as Fig. 1 shows. The ethanol extracts of the Fig. 2 are more active, since 3 of them, *C. trigyna*, *L. nepetaefolia* and *C. podolaena* significantly decreased the number of

Table 2: Cytotoxicity assessment of the 33 plant extracts on KB and Vero cell lines

	% of inhibition					
	DCM extracts		Ethanol extracts		Water extracts	
Plant	KB	Vero	KB	Vero	KB	Vero
L. nepetaefolia	32	20	13	14	0	8
M. pruinosa	16	8	0	0	0	8
S. uliginosa	50	20	8	18	14	0
H. ulmoides (bark)	24	23	30	0	8	0
H. ulmoides (leaves)	13	8	5	6	5	11
C. trigyna	0	13	0	19	0	11
C. podolaena	87	21	0	5	9	11
B. patula	12	5	14	2	0	8
U. lobata	8	5	9	9	7	13
M. scaber	17	9	21	17	17	2
T. rhomboidea	16	35	10	0	0	0

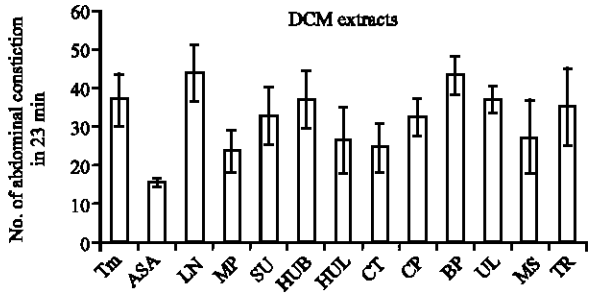

Fig. 1: Effect of the plants DCM extracts on the number of acetic acid-induced writhes. Tm: Control groups, DMSO 30%, ASA: Acetylsalicylic acid, 250 mg kg^{-1}, LN, MP, HUB, HUL, CT, CP, BP, UL, MS, TR = plant ethanol extracts, 250 mg kg^{-1}

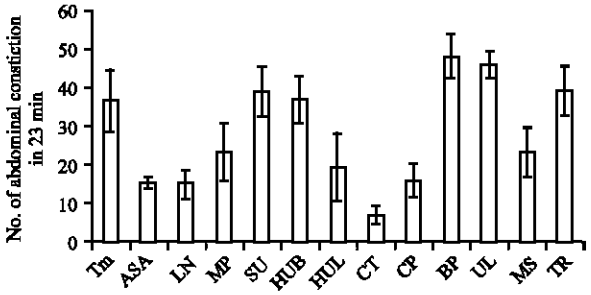

Fig. 2: Effect of the plants ethanol extracts on the number of acetic acid-induced writhes. Tm: Control groups, DMSO 30%, ASA: Acetylsalicylic acid, 250 mg kg^{-1}, LN, MP, HUB, HUL, CT, CP, BP, UL, MS, TR = plant ethanol extracts, 250 mg kg^{-1}

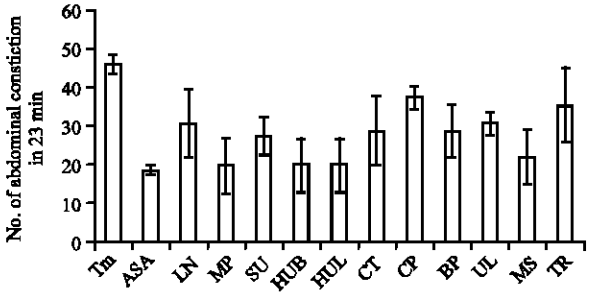

Fig. 3: Effect of the plants aqueous extracts on the number of acetic acid-induced writhes. Tm: Control groups, DMSO 30%, ASA: Acetylsalicylic acid, 250 mg kg^{-1}, LN, MP, HUB, HUL, CT, CP, BP, UL, MS, TR = plant ethanol extracts, 250 mg kg^{-1}

abdominal writhes induced by 0.6% acetic acid compared to the control groups. Moreover, the activity of *L. nepetaefolia* can be compared to ASA and *C. trigyna* is even more active than ASA. Figure 3 presents the values obtained with the 11 aqueous extracts. Except for *T. rhomboidea* and *C. podolaena*, all the aqueous extracts entailed a significant decrease of the number of abdominal contractions, comparatively to the control group. In decreasing order of efficiency, the aqueous extracts can be classified as follows: *M. pruinosa*>*H. ulmoides* (leaf and bark)> *M. scaber*>*S. uliginosa*> *B. patula*>*U. lobata*> *C. trigyna*>*L. nepetaefolia*.

By taking into account all the types of extracts, 8 plants on 10 have antinociceptive activities and the first three of them have activities that can compare with ASA. All the active extracts are aqueous or ethanolic extracts. These results confirm the relevance of the prescriptions of traditional medicine for the plants studied in this work. The acetic acid-induced writhing test chosen for this screening is very sensitive but little specific. It was appropriated to identify among our numerous extracts those having the best analgesic potential. However, to determine the mode of action of each active plant, it will be necessary to assess them via other peripheric and central analgesic tests.

Chemical basis for the analgesic activity: This study highlighted seven plants extracts displaying an analgesic potential similar to the potential of ASA or even higher. They were the ethanolic extracts of *Celosia trigyna* *Cogniauxia podolaena* and *Leonotis nepetaefolia* and the aqueous extracts of *Manotes pruinosa*, *Hymenocardia ulmoides* (bark and leaf) and *Mitracarpus scaber*. Though none of these plants had already been assessed for analgesic acticity, many of them belong to genera already known in the literature for their analgesic potential. Some of the plants had also been studied on other therapeutic targets. This allowed to isolate molecules which antinociceptive activity had already been assessed and proven. These already known compounds as well as the data of the TLC screening can give useful indications for the isolation of the compounds responsible for the activity of the extracts.

Celosia trigyna, which yielded the most active ethanolic extract (nearly twice the activity of ASA) had never yet been studied. The TLC screening confirmed the presence of terpenoids, with the addition of flavonoids, protein/amino acids, saponins and tannins. Another Celosia, *C. argentea*, presents a high antioxidant activity Odukoya *et al.* (2007) and Xue *et al.* (2006) isolated from an ethanol extract of *C. argentea*, β-sitosterol and stigmasterol. Since both these compounds are known for their antinociceptive activity (Santos *et al.*, 1995) they

could explain the plant activity, together with other terpenoids.

Leonotis nepetaefolia gave a very active ethanolic extract and a slightly active aqueous extract. This plant was already known for its antioxidative and anti-inflammatory activities (David *et al*., 2007; Parra-Delgado *et al*., 2004; Narukawa *et al*., 2001) but its analgesic potential was demonstrated for the first time in this study. The leaves of another *Leonotis*, *L. leonurus* displayed dose-dependent and significant antinociceptive effects against thermally and chemically induced pain stimuli in mice (Ojewole, 2005) and antioxidative activity (Oyedemi and Afolayan, 2011). The radical scavenging activity of *L. nepetaefolia* has been attributed to 4 glycosidic iridoids. One of them, verbascoside, has already proven its antinociceptive activity in several pain models (Backhouse *et al*., 2008) as well as is antioxidative activity (Narukawa *et al*., 2001; Takeda *et al*., 1999). Verbascoside is therefore at least partly responsible for the plant activity, probably with other compounds. Indeed, alongside volatile compounds, many other molecules were isolated from the aerial parts of *L. nepetaefolia*, among which stigmasterol. It can therefore be concluded that verbascoside and stigmasterol should contribute to the analgesic activity of *L. nepetaefolia.*

Cogniauxia podolaena's ethanolic extract was active but not its aqueous extracts. This plant was also the most cytotoxic, probably because of its terpenoids (like the cucurbitacins) and of its saponins (Banzouzi *et al*., 2008b). The TLC screening detected also flavonoids, lactones/esters, iridoids and anthraquinones. The high cytotoxicity and the hepatotoxicity found by Diatewa *et al*. (2002) are coherent with the recommendations of caution in the use of the plants issued by the traditional practitioners.

Manotes pruinosa, which yielded the most active aqueous extract, had never yet been studied for its analgesic potential. It has only been assessed for antiprotozoal and cytotoxic activities, with negative results (Mesia *et al*., 2008). The TLC screening revealed the presence of flavonoids, lactones/esters proteins/amino acids, iridoids, anthraquinones, saponins and tannins but it is difficult now to suggest which compounds may be responsible for the plant activity.

Hymenocardia ulmoides, giving the second most active aqueous extracts, had never yet been studied. The TLC screening indicated the presence of lactones/esters iridoids, terpenoids, saponins, tannins in both barks and leaves and flavonoids, anthraquinones and amino acids only in the leaves. The terpenoids will have to be studied more closely, because another *Hymenocardia, H. acida* presents antioxidant and anti-inflammatory activities (Mahmout *et al*., 2005; Sofidiya *et al*., 2006) and contains lupeol and β-sitosterol. Friedelin and derivatives may also contribute to the plant activity, since friedelin has been isolated from *H. wallichiana* (Yenjai *et al*., 2005) and presents analgesic activity (Isaias *et al*., 2004).

Mitracarpus scaber was known for its anti-inflammatory activity (Okoli *et al*., 2003). This study demonstrated that it has also a good analgesic potential. It contains ursolic acid (Gbaguidi *et al*., 2006) and kaempferol-3-O-rutinoside (Bisignano *et al*., 2000). Kaempferol-3-O-rutinoside` will have to be assessed for analgesic activity since kaempferol and some of its derivatives (kaempferol-3-O-beta-D-galactoside, kaempferol-3,7-O-alpha-dirhamnoside, kaempferol-3-O-glucoside) possess this activity (Toker *et al*., 2004; Orhan *et al*., 2007; Parveen *et al*., 2007). In another *Mitracarpus* species, *M. villosus* the analgesic activity was attributed partly to ursolic acid (Ekpendu *et al*., 2001). These data lead us to think that ursolic acid also explain a part of *M. scaber* activity probably with kaempferol derivates and maybe other compounds, since our chemical screening revealed the presence of iridoids.

Spilanthes uliginosa and *Brillantaisia patula*, both displaying a moderate activity, had never been assessed for analgesic activity. Flavonoids, lactones/esters, anthraquinones and saponins were detected in *S. uliginosa* leaf extracts and the screening confirmed the presence of iridoids and terpenoids, together with lactones/esters, anthraquinones and saponins in *B. patula* leaf extract. The flower extract of another *Spilanthes*, *S. acmella*, displayed an analgesic activity with the "tail flick" test. The active compound was isolated and determined as n-isobutyl -4,5- decadienamide (Ansari *et al*., 1988). As to the *Brillantaisia* genus, *B. palisotii* has been assessed for analgesic activity. Matheus *et al*. (2005) demonstrated that DCM, ethyl acetate and n-butanol extracts obtained from its stems had a spinal antinociceptive effect with similar patterns to all doses in both peripheral (acetic acid-induced writhing) and central (tail flick and hot plate) analgesic models. The chemical study of the aerial part of *B. palisotii* (Berrondo *et al*., 2003) revealed the presence of numerous molecules, among which α-amyrin, β-amyrin, sitosterol, stigmasterol, lupeol and verbascoside, all known for their antinociceptive activity (Nakamura *et al*., 1997; Villasenor *et al*., 2002; Santos *et al*., 1995; Martini *et al*., 2007).

Urena lobata and *Triumfetta rhomboidea* appear inactive in our study, but it may be interesting to assess

their activity with other tests. Indeed, analgesic compounds such as β-sitosterol, quercetin and friedelin derivatives have been isolated in *T. bartramia* (Ho *et al*., 1995) and the TLC screening showed in *T. rhomboidea* flavonoids, lactones/esters, terpenoids, iridoids, anthraquinones and saponins. As for *U. lobata*, the flavonoids of its flowers have been studied by Matlawska and Sikorska (1999) who found, among other compounds, kaempferol, quercetin and its derivatives. Quercetin is known since 1979 for its analgesic activity (Rylski *et al*., 1979) and more recent studies have demonstrated its action on central nervous system and determined its mechanism of action (Naidu *et al*., 2003). The TLC screening showed also the presence of flavonoids, lactones/esters, terpenoids, anthraquinones and saponins in the leaves.

CONCLUSION

The analgesic activity of eight plants from the traditional Congolese pharmacopoeia was demonstrated here for the first time and six of them yielded extracts as active as ASA. This necessary work opens the way for the research of the active molecules stemming from these plants as well as for their valuation as leads for the synthesis and the pharmacomodulation of compounds with analgesic potentiality. Compounds presenting the best selectivity indexes (low toxicity and high activity) will serve as chemical tracers in the manufacturing of new analgesic phyto-drugs.

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