Evaluation of the hepatotoxic and hepatoprotective effect of Rwandese herbal drugs on in vivo (guinea pigs barbiturate-induced sleeping time) and in vitro (rat precision-cut liver slices, PCLS) models

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Abstract

Precision-cut liver slices (PCLS) preserve the tissular organization of the organ and represent an in vitro model closer to in vivo conditions than hepatocytes cultures. As this may be an interesting tool not only for the investigation of hepatotoxic and protective effects but also for bioguided fractionations schemes, the usefulness of PCLS was compared with an in vivo test of liver function. Crude extracts derived from five herbs used in Rwanda for hepatoprotective activity were tested on CCl\textsubscript{4}-treated guinea pigs by the method of barbiturate-induced sleep modification. Aqueous extracts of \textit{Ocimum lamiifolium}, \textit{Crassocephalum vitellinum}, \textit{Guizotia scabra} and \textit{Vernonia lasiopus} leaves allowed animals to recover barbiturate sleep duration in proportions of 88\%, 78\%, 61\% and 34\%, respectively and \textit{Microglossa pyrifolia} was found inactive. Dried methanolic extracts of the 5 plants were then tested in vitro on rat PCLS for protection against acetaminophen-induced hepatotoxicity. In this model, \textit{G. scabra}, \textit{M. pyrifolia} and \textit{V. lasiopus} were found hepatotoxic by themselves and unable to prevent acetaminophen toxicity. The most active extract, obtained from \textit{O. lamiifolium}, was subjected to bioassay-guided fractionation by chromatography on Si–C\textsubscript{18} to yield two quite active fractions. From a single animal, at least 50 PCLS explants can be prepared, which allows testing large amounts of samples, strengthening ethnopharmacological data on hepatoprotective medicinal plants and investigating hepatotoxic effects.

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Keywords: Rat precision-cut liver slices (PCLS); Hepatotoxicity; Hepatoprotection; \textit{Ocimum lamiifolium}; \textit{Crassocephalum vitellinum}; \textit{Guizotia scabra}; \textit{Vernonia lasiopus}; \textit{Microglossa pyrifolia}; Guinea pigs; Sleeping time

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1. Introduction

The liver, a major organ of metabolisation and excretion, is susceptible to a number of pathologies, classified as cirrhosis, acute chronic hepatitis and hepatitis. Causes of hepatic trouble include parasitic and viral infections, autoimmune diseases and intoxication with various xenobiotics such as chlorinated solvents, alcohol, drugs, herbal medicines, peroxidized fatty acids, fungal toxins, industrial pollutants and radioactive isotopes (Evans, 2002). The predominant pathologies in a given country depend on the lifestyle and economic conditions. In Rwanda, viral hepatitis and their complications, cirrhosis and hepatic carcinoma, represent 80% of all liver pathologies, the 9th cause of morbidity (Musemakweli, 1999). A number of plants are traditionally used to treat liver diseases (Van Puyvelde, 1988). Except for vaccines and interferon α-2b, which concern only viral infections, modern medicine is quite limited in preventing or treating hepatic diseases; the only drugs available are choloagogues, choleretics, drugs for cholesterolic lithiasis, N-acetylcysteine and flavolignanes obtained from Silybum marianum. This limitation of therapeutic options gives considerable interest to the search for active compounds from plants traditionally used for these diseases (Evans, 2002). In this context, an ethnopharmacological inquiry into plants used as remedies for liver diseases was undertaken by our team in Rwanda, in 2004, interrogating a series of herbalists and traditional healers. These data were compared with published documents from Rwanda (Van Puyvelde et al., 1977; Van Puyvelde, 1988; Rwangabo, 1993) and other countries (Demissew et al., 1998; Vanhulle et al., 2001; Vickers and Fisher, 2002) which led to the selection of five herbs locally used for hepatoprotective activity (Table 1), Crassocephalum vitellinum, Guizotia scabra, Microglossa pyrifolia, Ocimum lamiifolium and Vernonia lasiopus. Such ethnopharmacological reports however do not give enough information regarding the clinical efficaciousness or safety of herbs and there are risks possibly associated with uncontrolled/ unsubstantiated use of herbal remedies. The present study is intended to explore whether these herbs could have protective effect on hepatocytes and to give an orientation to find hepatoprotective compounds that may be present in the extracts. This first step should eventually lead us to molecules responsible for the effect and further investigating mechanisms of action on the liver.

In order to study hepatotoxic and hepatoprotective compounds, i.e. agents capable to prevent and/or reverse the effects induced by a hepatotoxicant (CCl₄, α-galactosamine, acetaminophen, peroxides, etc.), a series of models have been developed either in vivo (histology, measurement of serum hepatic enzymes, barbiturate-induced sleeping time, prothrombine time and bromosulphaleine clearance) or in vitro (continuous cell lines and primary cultures of hepatocytes). Precision-cut liver slices (PCLS) preserve the tissular organization of the organ and represent an in vitro model closer to in vivo conditions than hepatocytes cultures (Morales et al., 1998; Vanhulle et al., 2001; Vickers and Fisher, 2004). As PCLS is not only an interesting tool for the investigation of hepatotoxic and protective effects but also for bioguided fractionation schemes, their usefulness has been further investigated on the five ethnopharmacologically selected Rwandan herbal drugs compared to an in vitro test of liver function, barbiturate-induced sleeping time.

2. Material and methods

2.1. Plant material

The leaves of C. vitellinum (Benth) S. Moore (Asteraceae), G. scabra (Vis) Chiov (Asteraceae), M. pyrifolia (Lam.) Kuntze (Asteraceae), O. lamiifolium Hochst ex. Benth (Lamiaceae) and V. lasiopus O. Hoffm (Asteraceae) were collected in the prefecture of Butare (South-Western Rwanda) in July 2004, air-dried in the shade and mechanically powdered. The plants were authenticated by Dr. M.-J. Bigendako, Center of Research in Phytomedicals and Life Sciences, Butare, Rwanda and voucher specimens were deposited in the BRLU herbarium, Belgium.

<table>
<thead>
<tr>
<th>Scientific name (family; organ)</th>
<th>Local name</th>
<th>Traditional usesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocimum lamiifolium (Lamiaceae; leaves)</td>
<td>Umusura</td>
<td>Hepatitis, gonorrhea, gastric ulcer asthma and dropsy</td>
</tr>
<tr>
<td>Crassocephalum vitellinum (Benth)</td>
<td>Isunu</td>
<td>Hepatitis, woman sterility, strong fever, dysmenorrhea, facilitates the deliverance of placenta, constipation and kids’ diseases</td>
</tr>
<tr>
<td>Guizotia scabra (Vis) Chiov (Asteraceae; leaves)</td>
<td>Ishikishike</td>
<td>Hepatitis, malaria, against helminthes</td>
</tr>
<tr>
<td>Vernonia lasiopus O. Hoffm (Asteraceae; leaves)</td>
<td>Ivumo</td>
<td>Hepatitis, wounds and kid’s constipation</td>
</tr>
<tr>
<td>Microglossa pyrifolia (Lam.) Kuntze (Asteraceae; leaves)</td>
<td>Umuhe</td>
<td>Hepatitis, malaria, rhumatism, gastric ulcer, wounds disinfection, eye treatment, head and abdomen pain</td>
</tr>
</tbody>
</table>

aData from the present work and from Van Puyvelde (1988).
2.2. Chemicals

Williams’ medium E (WME) and foetal calf serum (FCS) were purchased from Gibco BRL (Middlesex, UK), acetaminophen from Janssen Pharmaceutica (Beerse, Belgium), insulin (Actrapid HM) from Novo Nordisk (Bagsvaerd, Denmark) and gentamicine sulfate, carbon tetrachloride, pentobarbital, N-acetyl cysteine (NAC), β-glucuronidase, benzoazolinone chloroxazone and 6-hydroxychloroxazone from Sigma-Aldrich (Bornem, Belgium).

2.3. Extraction of plant material

2.3.1. Extracts for in vitro tests and phytochemical screening

Each plant (50 g) was macerated for 24 h and exhaustively percolated with solvents of increasing polarities, N-hexane, chloroform, methanol and water. The resulting extracts were evaporated to dryness (reduced pressure, 40 °C) yielding four crude extracts (Table 2), which were subjected to phytochemical analysis; the methanolic extract was also used for in vitro tests. 500 mg of the most active methanolic extract (O. lamiifolium) was submitted to column chromatography on silica gel C18 eluting with a gradient of water–methanol (100% water, 90:10, 80:20, 60:40, 50:50 and 100% methanol); 60 fractions of 10 ml were collected and combined upon thin-layer chromatography (TLC) analysis to yield 9 fractions (A–I).

2.3.2. Extracts for in vivo tests

Crude extracts were prepared for the 5 plants by a healer working in the dispensary of the Institute of Scientific and Technological Research of Butare, Rwanda, following his traditional protocol. Briefly, 350 g of each plant were infused in about 2000 ml of tap water, filtered on a cotton cloth and dried under reduced pressure at 40 °C to yield the extracts.

The traditional use of the five plants relies on aqueous decoctions and so only the polar extracts (aqueous decoction and methanolic leaves extracts) were selected for pharmacological testing.

Table 2. Extraction yields (m/m) for the 5 plants selected (50 g leaves + 500 ml solvent; sequential 24 h macerations with increasing polarity solvents).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Crassocephalum vitellinum</th>
<th>Guizotia scabra</th>
<th>Microglossa pyrifolia</th>
<th>Ocimum lamiifolium</th>
<th>Vernonia lasiopus</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane</td>
<td>1.4</td>
<td>1.6</td>
<td>2.5</td>
<td>3.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.6</td>
<td>4.9</td>
<td>5</td>
<td>3.2</td>
<td>41.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>9</td>
<td>13.9</td>
<td>18</td>
<td>6.4</td>
<td>8.4</td>
</tr>
<tr>
<td>Water</td>
<td>8.6</td>
<td>11.9</td>
<td>15.9</td>
<td>21.7</td>
<td>7.8</td>
</tr>
</tbody>
</table>

2.4. Animals

Male Wistar rats, weighing 250–300 g, were purchased from Iffa-credo (Les Oncins, France), housed in individual cages in a temperature- and light-controlled room (12 h dark/light cycles) receiving a standard diet AO3 (UAR, Villemoisson sur Orge, France) and water ad lib.

Male and female healthy guinea pigs, weighing 500–600 g, were raised in the Institute for Research, Science and Technology (IRST, Huye, Rwanda). Before the experiment, all guinea pigs were fasted for 16 h and, throughout the duration of in vivo experiments (3 days), they were fed with a thorough mixture of 2 edible leaves, Lactuca capensis (Thunb.) (Asteraceae) and Brassica oleracea (var. capitata) (Brassicaceae) and tap water. These plants are known to be edible vegetables; our experience shows that guinea pigs can be grown with this regimen without any apparent problem. As control animals were fed the same mixture, diet parameters should be under control. All experiments were conducted in accordance with international standards of animal welfare as recommended by the European Union on Animal Care (CCE Council 86/609).

2.5. Measurement of hepatoprotective activity

2.5.1. In vitro tests on acetaminophen-treated precision-cut liver slices

Surgical procedures were carried out on fed rats under pentobarbital (60 mg/kg) anaesthesia. The liver was perfused in situ with ice-cold Krebs–Ringer solution before slicing. PCLS (250–300 μm thick) were prepared from the whole liver without distinction of lobes in oxygenated ice-cold Krebs–Ringer buffer using a Krumdieck tissue slicer according to procedures previously described (Evdokimova et al., 2001). They were stored 30 min at 4 °C in WME containing FCS (10% v/v), glutamine (2 mM), insulin (100 nM) and dexamethasone (10 nM) and then transferred to 25-ml vials containing WME (2 slices/4 ml) supplemented with glutamine (2 mM), insulin (100 nM) and gentamicin sulfate (50 μg/ml). PCLS were incubated in a shaking water
bath (100 cycles/min) at 37°C under a continuous flow of O₂–CO₂ (95%:5%) and randomized to avoid any variability between slices that may come from localization in liver lobes or size. After 1 h of preincubation, allowing fresh slices to recover ATP content, the slices were transferred to other vials containing fresh medium and supplemented with different combinations of acetaminophen (hepatotoxic agent, 10 mM), N-acetyl cysteine (hepatoprotective agent, 20 mM) and/or plant extracts (1 mg/ml) for 24 h. Unsupplemented control slices were sampled after this 24 h incubation (control). As the PCLS test is intended for bioguided fractionation (i.e. the use of a bioassay to guide the purification and isolation of active compounds), with relatively low amounts of material to be purified, a preliminary study led us to select a sensibly low dosage for tested extracts (1–5 mg/ml); this is the same order of magnitude as for acetaminophen (1.5 mg/ml) and NAC (3.3 mg/ml).

Liver slices were washed twice in saline and sonicated immediately in 1 ml of 2% perchloric acid. The intracellular ATP content was measured on neutralized perchloric acid extracts using the ATP Bioluminescence Assay Kit CLS II from Boehringer–Mannheim (Germany). Protein measurement was performed in duplicate on sonicated PCLS by the method of Lowry et al. (1951) using bovine serum albumin as standard (Lowry et al., 1951).

For each incubation condition, 2 slices were tested with 2 ATP measurements per slice; samples were compared by ANOVA and pairwise comparisons were performed by a t-test with Bonferroni correction. For histological examination, control and treated PCLS were immersed in 10% formaldehyde, embedded in paraffin, sliced with a microtome, layered on microscope slides and stained with haematoxyline–eosine. CYP2E1 activity was measured by incubation of PCLS with chlorzoxazone and HPLC measurement of its hydroxylation products using the ATP Bioluminescence Assay Kit CLS II from Boehringer–Mannheim (Germany). Protein measurement was performed in duplicate on sonicated PCLS by the method of Lowry et al. (1951) using bovine serum albumin as standard (Lowry et al., 1951).

For each incubation condition, 2 slices were tested with 2 ATP measurements per slice; samples were compared by ANOVA and pairwise comparisons were performed by a t-test with Bonferroni correction. For histological examination, control and treated PCLS were immersed in 10% formaldehyde, embedded in paraffin, sliced with a microtome, layered on microscope slides and stained with haematoxyline–eosine. CYP2E1 activity was measured by incubation of PCLS with chlorzoxazone and HPLC measurement of its hydroxylation metabolite, 6βH-chlorzoxazone (Wauthier et al., 2004). Briefly, 4 slices per experimental condition in 2 ml medium were added with 20 μl of 10 mg/ml chlorzoxazone in methanol and incubated (37°C, 90 min); 300 μl of medium were added with 1.21 IU β-glucuronidase in 100 μl acetate buffer (0.3 M, pH 5) and further incubated (37°C, 2 h). Upon addition of 150 μl ZnSO₄ (15% in water) and 100 μl benzoazolinone (0.1 mg/ml in methanol, internal standard) and centrifugation (13000g, 1 min), the supernatant was filtered (PVDF 4 mm, 0.45 μm) and analyzed by HPLC in the following conditions: column C₁₈ BDS 3 μm (LC Packings); mobile phase, ammonium acetate (3.9 g/l, pH 4.25)–acetonitrile (84:16) at 1.5 ml/min; UV detection at 287 nm.

2.5.2. In vivo tests on CCl₄-treated male and female guinea pigs

Guinea pigs were divided into groups of 8 animals each, including a control group (no treatment prior to determination of sleeping time). Animals were inspected every 12 h for humane endpoints, including behaviour (abnormal movements and immobility), clinical signs (swollen abdomen, signs of severe distress, diarrhoea and convulsions) and eventual signs of impending death (convulsions, lateral position, recumbence and tremor). None of the animals had to be withdrawn from the study during the 3-day experiments. At day 1, the CCl₄ group was given orally 1.5 mg/kg carbon tetrachloride as a fresh mixture with an equal volume of peanut oil. The duration of the sleep was determined two days after the administration of carbon tetrachloride (day 3). In this model, pure compounds have previously been tested from 200 to 800 mg/kg (Van Puyvelde et al., 1989). Reasoning that the extracts are most probably mixtures of active and inactive compounds, with unknown bioavailability, we selected intraperitoneally (i.p.) a dose of 1 g/kg.

At day 1, the treated groups received the preparation orally in doses of 1 g extract/kg 1 h prior to carbon tetrachloride. On the second and the third day, the same dosage of the preparation was given and the duration of sleep determined on the third day, 30 min after the administration of the last preparation. Animals received pentobarbital intraperitoneally in doses of 50 mg/kg (the barbiturate was dissolved in 0.1 N NaOH). For each animal, sleep duration was defined as the time between loss and recovery of righting reflex.

2.6. Preliminary phytochemical analysis

Phytochemical screening of plants was undertaken using classical methods (Rwagabo, 1986; Wagner and Bladt, 1996). The screening covered (i) alkaloids (Mayer and Dragendorff’s reagents; silica gel TLC in toluene–ethyl acetate–diethylamine (70:20:10) with spraying of Dragendorff and iodoplatinate reagents); (ii) terpenoids–sterols (Liebermann’s reagent; silica gel TLC in toluene–chloroform–ethanol (40:40:10) with spraying of anisaldehyde–sulphuric acid); (iii) flavonoids (Shinoda’s reagent); (iv) anthocyanins (HCl 2 N); (v) saponins (honeycomb froth persisting for at least 30 min after vigorous shaking of the water extract; Liebermann–Buchard’s test); (vi) tannins (ferric chloride reagent and gelatine-salt block test) and (vii) anthraquinones (Borntrager’s reagent).

3. Results

C. vitellinum, O. lamiifolium, G. scabra, M. pyrifolia, and V. lasiopus were selected based on ethnopharmacological inquiries and because of their use in liver diseases (Table 1). The leaves of the five plants were extracted with different solvents and their extraction yields are
O. lamiifolium compounds in the tested extracts. Fractionation of mechanism of action or a lower dose of active efficient than NAC, which may come from a different toxic quinoneimine metabolite (NAPQI). The NAC forms a nucleophilic adduct with the electrophilic efficient in preventing acetaminophen damage; indeed, NAC, widened sinusoidal spaces and chromatolytic nuclei. acetaminophen induces altered and necrotic cells with presented in Table 2. Each plant was screened for the main classes of phytochemicals (Table 3) which revealed the presence of alkaloids, flavonoids, terpenoids–steroids, saponins and tannins as major chemical constituents.

Figs. 1 and 2 compare the effects of the five different methanolic extracts with NAC as a reference hepatoprotective agent in acetaminophen-challenged PCLS incubated in Williams’medium either with or without paracetamol. On PCLS G. scabra, M. pyrifolia and V. lasiopus were found hepatotoxic by themselves (reduction in ATP levels, 68.7%, 89.7% and 88.3%, respectively) and unable to prevent acetaminophen toxicity (Fig. 1). C. vitellinum and O. lamiifolium were not hepatotoxic and protected the liver slices against the induced hepatotoxicity (Figs. 1 and 2). NAC proved extremely efficient in preventing acetaminophen damage; indeed, NAC forms a nucleophilic adduct with the electrophilic toxic quinoneimine metabolite (NAPQI). The C. vitellinum and O. lamiifolium extracts were comparatively less efficient than NAC, which may come from a different mechanism of action or a lower dose of active compounds in the tested extracts. Fractionation of O. lamiifolium by column chromatography on C18-modified silica gel and elution by gradient water:methanol yielded 9 subfractions, Ocimum A–I, that present different TLC profiles. These were similarly tested on PCLS (data not shown), but only the first 2 fractions were found to prevent acetaminophen toxicity (Fig. 2). The experiment was repeated on a series of animals for the extracts yielding a positive response (Table 4). The histological examination of slices (Fig. 3) shows that acetaminophen induces altered and necrotic cells with widened sinusoidal spaces and chromatolytic nuclei. NAC, C. vitellinum and O. lamiifolium extracts protect the tissue against acetaminophen toxicity, nuclei being apparent on a dense and coloured cytoplasm with normal sinusoidal spaces. Acetaminophen toxic metabolite, N-acetyl-p-benzoquinoneimine, is mainly produced by the cytochrome CYP2E1; the influence of active extracts on CYP2E1 was investigated by measuring the metabolization of a tracer compound, chlorozoxazone (Table 5). Whereas O. lamiifolium fraction A had no effect by itself on control slices, the C. vitellinum methanolic extract slightly reduced CYP2E1 activity, which may partly account for its protective activity. The formation of 6OH-chlorozoxazone was severely decreased by acetaminophen treatment (3% vs. control), which could partly be prevented by C. vitellinum methanolic extract (40%) and by O. lamiifolium fraction A (51%). These preliminary data however show no dose-effect relationship and need further investigation.

In in vivo studies, liver injury induced by CCl4, the best characterized system of xenobiotic-induced hepatotoxicity, is a commonly used model for screening the anti-hepatotoxic/hepatoprotective activity of drugs (Brattin et al., 1985; Williams and Burk, 1990; Recknagel, 1991). As pentobarbital is metabolized exclusively in liver, the sleeping time after a given dose is a measurement of hepatic metabolism. After poisoning with carbon tetrachloride, liver damage causes a sleeping time increase (Table 6); in the presence of an hepatoprotective agent, sleeping time is wholly or partly restored (Wagner and Wolff, 1977). M. pyrifolia aqueous extract does not protect animals from CCl4 toxicity; protection afforded by V. lasiopus was extremely modest (34.1%). G. scabra, C. vitellinum and O. lamiifolium allowed recovering sleeping time by 61.4%, 77.9% and 88.2%, respectively. These data partly confirm those obtained on PCLS with methanolic extracts in which G. scabra, M. pyrifolia and V. lasiopus were found hepatotoxic by themselves whereas C. vitellinum and O. lamiifolium protected the liver slices against the acetaminophen hepatotoxicity.

### Table 3. Results of the phytochemical screening.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Alkaloids</th>
<th>Terpenoids and steroids</th>
<th>Flavonoids</th>
<th>Anthocyanins</th>
<th>Saponins&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tannins</th>
<th>Anthraquinones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C</td>
<td>P</td>
<td>H</td>
<td>C</td>
<td>M</td>
<td>W</td>
</tr>
<tr>
<td>C. vitellinum</td>
<td>++/−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>G. scabra</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>M. pyrifolia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>O. lamiifolium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>V. lasiopus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup>In brackets, height of foam column. 
<sup>b</sup>Tested material (P = powder, H = N-hexane extract, M = methanol extract, W = aqueous extract). 
<sup>c</sup>Shinoda’s reagent: orange coloration (flavones). 
<sup>d</sup>Shinoda’s reagent: violet coloration (flavonones). 
<sup>e</sup>Shinoda’s reagent: red coloration (flavonols).
4. Discussion

In contrast to many other pathologies, the symptomatology of a number of hepatic troubles (icterus) is evident for traditional healers, who can easily evaluate the response to treatments and thus probably select efficient herbal medicines. Many studies have already led to the characterization of more than 170 constituents isolated from 110 plants belonging to 55 families, including terpenoids, curcuminoids, lignoids and flavonoids and cyanogenetic glycosides (Luper, 1998, 1999; Evans, 2002; Seef et al., 2002; Thyagarajan et al., 2002) and a large number of plants from India, China and Europe have been reported to treat liver diseases and boost liver functions (*Curcuma longa*, *Picrorrhiza kurroa*, *Camellia sinensis*, *Glycyrrhiza glabra*, *Bupleurum falcatum*, *S. marianum*, *Taraxacum officinale*, etc.) (Kiso et al., 1984; Sugiyama et al., 1998; Ram, 2001; Evans, 2002; Thyagarajan et al., 2002), including some species related to those of the present study, *O. gratissimum*, *O. sanctum* and *C. crepidioides* (Chattopadhyay et al., 1992; Effraim et al., 2002; Aniya et al., 2005). In contrast, only relatively scarce data are available on the African traditional pharmacopoeia and the five plants we selected for this study, according to ethnobotanical inquiries, have not been previously investigated for hepatoprotective activity.

Experimentally, the concept of liver protection strongly depends on the chosen toxicant and laboratory animal species, which may lead to the selection of plant extracts and compounds active on a given model but without clinical relevance. To maximize the chances to select active extracts, two biological models, *in vitro* tests on acetaminophen-treated rat liver slices and *in vivo* tests on carbon tetrachloride-challenged guinea pigs, were selected for their different toxicity mechanistics. CCl₄ is

![Fig. 1. Effect of N-acetylcysteine (20 mM) and the methanolic extracts of *Ocimum lamifolium*, *Guizotia scabra*, *Microglossa pyrifolia* and *Vernonia lasiopus* (1 mg/ml) on acetaminophen-challenged (10 mM) and control precision-cut liver slices. Mean + SD of duplicate ATP measurements on 2 PCLS obtained from the same rat; ***: p < 0.001, NS: p > 0.05 (ANOVA with post-hoc pairwise comparisons; t-test with Bonferroni correction).](image-url)
metabolized by CYP2E1 in an extremely reactive radical, CCl₃• that peroxidizes lipids, inducing hepatic lesions (Plaa and Hewitt, 1998). Acetaminophen (paracetamol), a frequently used analgesic and antipyretic drug, is primarily metabolized by sulfation and glucuronidation to unreactive metabolites, whereas a minor but highly reactive N-acetyl-p-benzoquinoneimine metabolite is normally detoxified by glutathione. Following

**Fig. 2.** Effect of N-acetylcysteine (20 mM), of *Ocimum lamifolium* and *Crassocephalum vitellinum* leaves methanolic extracts and of 2 fractions obtained from the *O. lamifolium* extract (1 mg/ml) on acetaminophen-challenged (10 mM) and control precision-cut liver slices. Mean ± SD of duplicate ATP measurements on 2 PCLS obtained from the same rat; ***: *p* < 0.001, NS: *p* > 0.05 (ANOVA with post-hoc pairwise comparisons; *t*-test with Bonferroni correction).

**Table 4.** Effect of N-acetylcysteine (20 mM) and the methanolic extracts of *Ocimum lamifolium* and *Crassocephalum vitellinum* (1 mg/ml) on acetaminophen-challenged (10 mM) precision-cut liver slices from different animals.

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>ATP (nmoles/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
</tr>
<tr>
<td>Acetaminophen</td>
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</tr>
<tr>
<td>Acetaminophen + NAC</td>
<td>12</td>
</tr>
<tr>
<td>Acetaminophen + <em>Crassocephalum vitellinum</em></td>
<td>6</td>
</tr>
<tr>
<td>Acetaminophen + <em>Ocimum lamifolium</em></td>
<td>10</td>
</tr>
<tr>
<td>Acetaminophen + <em>Ocimum lamifolium</em> fraction A</td>
<td>4</td>
</tr>
</tbody>
</table>

*a* 2 slices per animal.

*Kruskal–Wallis post-hoc pairwise comparisons versus treatment acetaminophen (Bonferroni correction; ***p < 0.001; *p < 0.05)."
overdosage, the glutathione pool gets depleted and this metabolite accumulates to produce liver injury (Kupeli et al., 2006). In vitro studies on acetaminophen-treated PCLS show that N-acetylcysteine, a major antidote in clinical cases of acetaminophen intoxications, prevents depletion of ATP; NAC also offers a hepatoprotective

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**Table 5.** CYP2E1 activity of acetaminophen-challenged (10 mM) and control precision-cut liver slices upon treatment with N-acetylcysteine, *Crassocephalum vitellinum* (methanolic extract) and *Ocimum lamiifolium* (fraction A) (triplicate measurements on 4 PCLS obtained from the same rat; RSD = 6.2%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-OH-chlorzoxazone</th>
<th>% vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (24 h incubation)</td>
<td>10.1</td>
<td>100</td>
</tr>
<tr>
<td>C. vitellinum (2 mg/ml)</td>
<td>7.4</td>
<td>73</td>
</tr>
<tr>
<td>C. vitellinum (5 mg/ml)</td>
<td>9.5</td>
<td>94</td>
</tr>
<tr>
<td><em>O. lamiifolium</em> fraction A (2 mg/ml)</td>
<td>10.1</td>
<td>100</td>
</tr>
<tr>
<td><em>O. lamiifolium</em> fraction A (5 mg/ml)</td>
<td>9.6</td>
<td>95</td>
</tr>
<tr>
<td>Acetaminophen (10 mM)</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>Acetaminophen + C. vitellinum (2 mg/ml)</td>
<td>3.4</td>
<td>34</td>
</tr>
<tr>
<td>Acetaminophen + C. vitellinum (5 mg/ml)</td>
<td>4.1</td>
<td>40</td>
</tr>
<tr>
<td>Acetaminophen + <em>O. lamiifolium</em> fraction A (2 mg/ml)</td>
<td>5.2</td>
<td>51</td>
</tr>
<tr>
<td>Acetaminophen + <em>O. lamiifolium</em> fraction A (5 mg/ml)</td>
<td>5.2</td>
<td>51</td>
</tr>
</tbody>
</table>

---

**Table 6.** Effect of the aqueous decoctions on the duration of pentobarbital-induced sleeping time (CCl4-intoxicated guinea pigs).

<table>
<thead>
<tr>
<th>Plants</th>
<th>Sleeping time (ST) (min) (mean ± SD; n = 8)</th>
<th>% recoveryb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pentobarbital (60 mg/kg)</td>
<td>144 ± 3</td>
<td>–</td>
</tr>
<tr>
<td>Control CCl4 (Hepatotoxicant, 1.5 ml/kg)</td>
<td>300 ± 5</td>
<td>–</td>
</tr>
<tr>
<td><em>Crassocephalum vitellinum</em></td>
<td>179 ± 6***</td>
<td>77.9</td>
</tr>
<tr>
<td><em>Guzotia scabra</em></td>
<td>204 ± 7***</td>
<td>61.4</td>
</tr>
<tr>
<td><em>Microglossa pyrifolia</em></td>
<td>297 ± 6NS</td>
<td>1.7</td>
</tr>
<tr>
<td><em>Ocimum lamiifolium</em></td>
<td>162 ± 5***</td>
<td>88.2</td>
</tr>
<tr>
<td><em>Vernonia lasiopus</em></td>
<td>247 ± 5***</td>
<td>34.1</td>
</tr>
</tbody>
</table>

Treatments were compared by ANOVA with post-hoc pairwise comparisons (t-test with Bonferroni correction; ***: p<0.001, NS: p>0.05).

b% recovery = (Hepatotoxicant ST-Antihepatotoxicant ST) × 100.
effect against other toxins known to deplete cellular ATP levels (Popat et al., 2002).

As both animal species investigated (rat and guinea pig) have different sensitivity to toxic action of chemicals and significant differences in xenobiotics metabolism, this widens the research for possible hepatoprotective mechanisms and compounds. However the ultimate challenges remain (i) the translation from hepatoprotection to a potential beneficial effect on chronic diseases such as viral hepatitis or cirrhosis, which is still quite undocumented and (ii) the validation of such data on humans. On our 2 models, *O. lamiifolium* and *C. vitellinum* polar extracts were found particularly active in contrast to *M. pyrifolia* (no activity on both models) and *V. lasiopus* (modest activity only on the in vivo model). A discrepancy was observed between models for the extracts of *G. scabra*. This may come from differences in toxicant activity mechanism, in the composition of tested extracts and/or in doses.

*O. lamiifolium* methanolic extract is already known for antipyretic activities in mice (Makonnen et al., 2003) and for the presence of a major flavonol O-glycoside, the quercetin 3-O-xylene galactoside, and large amounts of flavone 5-O-glycosides (Gray et al., 2002). *C. vitellinum* and *G. scabra* proved active on ileon and uterus of guinea pigs and on the arterial pressure of rabbits (Chagnon, 1984). Sesquiterpene lactones and other terpenoids were reported from these two herbs (Zdero et al., 1991; Zollo et al., 2000). *V. lasiopus* is known for antiulcerous and analgesic activities (Johri et al., 1995). Some glucosides with antibacterial activity and antimalaric terpenoids have been isolated from *M. pyrifolia* (Rucker et al., 1994; Köhler et al., 2002).

The hepatoprotective ability of *O. lamiifolium* and *C. vitellinum* polar extracts may be connected with their flavonoids, since these phytochemical constituents, detected in our active extracts, have been implicated as hepatoprotective factors in *C. crepidioides* (Asteraceae) on CCl₄-induced toxicity (Aniya et al., 2005) and in *Equisetum arvense* L. (Equisetaceae) on tacrine-induced toxicity in Hep G2 cells (Hyuncheol, 2004).

Further work is in progress to determine if any of these compounds may be responsible for the hepatoprotective activities of *C. vitellinum* and *O. lamiifolium*.

To the best of our knowledge, this is the first time that the PCLS model is proposed and used for the bioguided fractionation of active extracts. PCLS indeed represent an attractive model in the study of natural products; a fair number of tests were carried out on the explants of a single animal. The PCLS however present the drawback of not being perfused, the liver slices simply bathing in the nutrient medium. Compared to in vivo conditions, in which the liver is continually perfused by blood circulation, sensibly higher concentrations of test compounds have to be used consequently. Compared to in vitro tests on monolayer cell cultures, higher concentrations of tested compounds are also required as cells inside the tissue need the diffusion of compounds through external cell layers (including external layers of dead cells induced by the slicing process). Despite this shortcoming, the PCLS model allows strengthening of ethnopharmacological data on hepatoprotective medicinal plants and investigating the hepatotoxic effects while sensibly reducing the number of experimental animals.

Our results support the Rwandese traditional use of four of the five plants as antihapatitis remedies. They suggest in vivo hepatoprotective effects of *O. lamiifolium*, *C. vitellinum*, *G. scabra* and *V. lasiopus* aqueous extracts. Given the direct toxicity of *G. scabra*, *M. pyrifolia* and *V. lasiopus* methanolic extracts on the liver slices model, these three herbs should be further evaluated. Their eventual use in traditional medicine should be quite cautious and accompanied by a monitoring of the hepatic function. It is noteworthy that the two herbs that proved active and nontoxic in our models, *C. vitellinum* and *O. lamiifolium* leaves, are edible vegetables or food additives in Tanzania (Copeland, 2004) and Ethiopia (Demissew and N., 1994), respectively. This gives some more indications of a probably safe medicinal use. Carrying out further studies on the active compounds of *O. lamiifolium* and *C. vitellinum* in order to assess their structure and elucidate their mechanism of action is worthy.

**Acknowledgements**

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