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Effects of *Lycopodium clavatum* and *equisetum arvense* extracts from western Romania

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Abbreviations: ALT–alanin transaminases, AST–aspartate transaminases, GC-MS–gas chromatograph coupled with mass spectrometry.

Abstract

Plants have always excited interest because of their active principles that could be a source of healing in various affections. The aim of this study was to demonstrate that the hepatoprotective and antimicrobial effects of Lycopodium clavatum and Equisetum arvense from the Western parts of Romania (Arad County) are not as pronounced as described in literature, against xenobiotic intoxication or microbial infection. To identify the plants active compounds, hydroalcoholic extracts were analyzed by gas chromatography coupled with mass spectrometry. Hydroalcoholic extracts of these plants were administered to acetaminophen induced liver injury mice model. Liver functions were investigated by transaminases, immunohistological, structural and ultra-structural analyses. Hydroalcoholic plant extracts were tested for antimicrobial activity. Results show that hydroalcoholic plant extracts had only moderate hepatoprotective and antimicrobial effects, conclusion sustained by the transaminases serum values. Histological and ultrastructural analyses did not show a reduced damage of the extracts treated groups compared to the acetaminophen treated group. Microbiology analyses showed that plant extracts induced a moderate bacteriostatic effect on the growth of most of the tested bacteria species, but not against fungi. By this study we have added new insights about these local plants medicinal potential.

Keywords: acetaminophen; clubmoss; horsetail; hepatoprotective; antimicrobial.

1. Introduction

Plants are today, as were in ancient times, a natural source of drugs and supplements, but pharmaceuticals remain the principal mean of treatment in all diseases and affections in most countries. Many chemical substances and drugs ingested accidentally or intentionally affect the liver because it is the most metabolically active organ and is involved in most of the body's metabolic functions, in the secretion of bile, and excretion and resorption of hepatic metabolites

(Bloom and Fawcett [1]). Synthetic drugs are considered xenobiotic substances by the liver and are treated accordingly, as possible intoxicants, thus the need to use natural products that have curative and beneficial actions, such as are local spontaneous plants (Adewusi and Afolayan [2]).
Two plants were described in literature to have hepatoprotective potential: *Lycopodium clavatum* and *Equisetum arvense*. Both can be found in the wet woods and pastures on hills and mountains, or in the crops (*E. arvense*) from Europe to Asia or America. *L. clavatum* Linn (**Lycopodiaceae**), also known as clubmoss, is a perennial herb with thin adventitious roots, long forked crawling stem and many thin short linear leaves (Ardelean and Mohan [3]). *L. clavatum* herba contains as active principles clavatin, annotin, lycopodine, nicotine triterpens, flavonoids and minerals (Ardelean and Mohan [3]). Orhan [4]) found in clubmoss extracts: dihydrocaffeic acid, vanillic acid, p-hydroxybenzoic acid, syringic acid, p-coumaric acid, and in another report found lycopodine in 84.5% proportion as the most abundant of alkaloids (Orhan [4]). Traditionally, this plant is used for treating alcohol and smoking addictions, and also liver and lung conditions (Ardelean and Mohan [3]). A recent study showed that *L. clavatum* extracts can be used for antimicrobial purposes against certain bacteria and fungi strains, and it can have antiviral effects as well, all due to the presence of the alkaloids mentioned above (Orhan [4]; Ndip [5]).
E. arvense Linn. (**Equisetaceae**) is also a perennial herb with a fertile stem emerging in the spring and an infertile bushy green stem that appears during summer time. Studies showed that the infertile photosynthesizing horsetail contains silicic acid, equisetonin, isoquercetin, metoxypiridin, nicotine, palustrin, palustridin, saponin, articulatidin, gluteolin, salicilic acid, malic acid, fatty acids, minerals, vitamin C (Milovanovic [6]). The horsetail essential oil contains timol, cineol, linalool, hexahydropharnesil, cis-geranil acetone and trans-ionone (Radulovic [7]). In a study made by Oh [8] it was proven that the hepatoprotective substances are onitin and luteolin. Extracts have anti-proliferative (Wang [9]) and soothing effects (Rezaie [10]). Milovanović [6] and Radulović [7] showed antibacterial effects of different solvent extracts and oil extracts against frequent pathogens.
Many recent studies have shown some beneficial activity of these plants, whether they are good antioxidants (Milovanovic [6]), possible good citostatics or they have a soothing and healing effect (Pattewar [11]; Nagori [12]). Some studies show a certain protective activity against liver induced carcinomas (Oh [8]). Authors extracted plant products in a range of solvents: water, ethanol, methanol, dichloromethane, petroleum ether, chloroform, ethyl acetate, n-butanol, out of which some were used for animal testing (Suciu [13]).
The purpose of our study was to evaluate the hepatoprotective and antimicrobial effects of hydroalcoholic extracts of two plants: *Lycopodium clavatum* and *Equisetum arvense* that spontaneously grow in the Western parts of Romania. For the GC-MS analysis we used four types of extraction solvents: ethanol, methanol, dichloromethane and hexane. Only the hydro-ethanolic extracts were used for antimicrobial activity evaluation and for *in vivo* studies of hepatoprotective effects on mice with acetaminophen-induced liver injury. Mice livers were evaluated for hepatic lesions by biochemical, histological, immunohistological and ultra-structural analyses and we have seen no significant hepatoprotective activity for whole extracts of *L. clavatum* and *E. arvense*.

2. Materials and Methods

Plant collection

L. clavatum aerial parts were collected from the neighbourhood of Hălmăgel and *E. arvense herba* from Cladova and Iosaş localities, in the Zărand Mountains (Romanian Western Carpathians) and Western Plain, Arad County. The harvest was done in August and September 2011 and the plants were shade dried. The plants were determined by a taxonomy specialist and voucher specimens were deposited at the "Vasile Goldis" Western University of Arad Herbarium for future reference.

Extracts

The finely milled dried plants were macerated in a proportion of 1:20 (w/v), 0.5 g *L. clavatum* with 10 mL of 80% ethanol, and 0.5 g *E. arvense* with 12 mL 80% ethanol for 24 hours with continuous mixing. The resulting extract was filtered and evaporated to dryness under nitrogen, weighted and re-suspended in 6.5 mL 40% ethanol, methanol, dichloromethane and hexane. The extraction protocol was the same for all extracts and adapted after similar previous studies (Pathak [14]; Milovanovic [6]; Pathak [15]). The extracts were used for gavage, gas chromatography-mass spectrometry (GC-MS) analysis, and microbiology studies. Methanol, dichloromethane and hexane extracts were used for GC-MS evaluation and only the hydro-ethanolic extracts were used for mice administration and microbiology assessment.

Animal model

We used young NMRI mice, with *ad libitum* access to food and water, housed in controlled temperature (22-24ºC) and 12 h light/dark cycle with lights on at 7:00 am. All animal experiments comply with European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986). The University of Medicine and Pharmacy Timisoara Ethical Committee approved the experimental protocol. Mice were injected intraperitoneally with a single dose of 400 mg/kg acetaminophen (1.2 mL/dose) (Sigma-Aldrich) and were grouped in 10 lots (12 mice/lot) as follows: a control group, an acetaminophen treated group, two groups treated with acetaminophen and 300 mg dry extract/kg body weight of 40% ethanolic clubmoss extract, respectively 40% ethanolic horsetail extract, and another two groups treated with 3 g dry plant extract/kg of body weight, a relative control group treated with 40% ethanol, a relative control group treated with 40% ethanol and acetaminophen, and two relative control groups treated with the 300 mg of dry plant extract/kg of body weight alone. Treatments were administered by gavage, in 400 µl volume doses, for three consecutive days after the acetaminophen administration, and in the fourth day the mice were sacrificed by cervical dislocation under ether anaesthesia. The intoxication model was adapted from Pathak [15]. Serum and liver samples were collected for further analyses.

GC-MS analysis

1 µL sample was injected in a HP6890 Series Gas Chromatograph coupled with a Hewlett Packard 5973 Mass Selective Detector. The gas chromatograph was equipped with a split–splitless injector and a Factor Four Capillary Column VF-35ms fused silica column of 35% phenyl-methylpolysiloxane, 30 m x 0.25 mm, film thickness 0.25 µm. The GC conditions include

a temperature range of 50–250°C with 4°C/min, and a solvent delay of 5 min. The injector was maintained at 250°C, helium was the GC carrier gas at 1.0 mL/min and the sample was injected in the splitless mode. The MS conditions were as follows: ionization energy, 70 eV; electronic impact ion source temperature, 200°C; quadrupole temperature, 230°C; scan rate 1.6 scans/s; mass, 40–500 amu. For the identification of the compounds, the mass spectra of the samples were compared with those of the NIST/EPA/NIH Mass Spectral Library 2.0. The peak areas of compounds were summed to 100% and the percentage area corresponding to each compound was plotted.

Measurement of total protein concentration

Liver samples were homogenized in phosphate buffer saline (1:1) and protein concentration was made using Bradford method. The standard curve was obtained using bovine serum albumin (BSA) (Bradford [16]).

AST and ALT analysis

The blood was collected by cardiac puncture, left to clot for 30 minutes, centrifuged and processed according to Siemens Diagnostics ALT-AST Kit.

Histological analysis

Mice livers were washed with saline buffer and fragments were collected from the same area of the liver for all individuals. The specimens were fixed in 4% formaldehyde, embedded in paraffin, cut into 3 µm thick slices, and were attached to glass slides, kept for an hour in 50°C toluene and rehydrated in successive 5 minutes baths of cold toluene, pure alcohol, 90% alcohol, and water. Rehydrated liver slices were stained with haematoxylin, washed with water and dehydrated following the reversed steps of hydration. The slides were then mounted with hydrophobic mounting media.

Immunohistological analysis

Liver fragments were processed as described above, except that after rehydration the slices were boiled for 15 minutes in an antigen retrieval solution then left to gradually cool to room temperature. The immunolabeling was made using the following primary antibodies: anti-cyclooxygenases 1 and 2 (COX1 and COX2, 1:500 dilutions), anti-lipooxygenases 5, 12 and 15 (LOX5, 12, and 15, 1:200 dilutions), anti-ED1 (CD68) and anti-ED2 (CD163), dilution 1:250. The secondary antibody used was biotinilated and the substrate was diaminobenzidine (DAB). For the negative control we omitted the primary antibody.

Electron Microscopy analysis

The liver specimens were fixed in 2.7% glutaraldehyde and 2% osmium tetraoxide, successively washed in phosphate buffer and dehydrated in increasing concentration of acetone, embedded in epon, and cut in 50-90 µm thin slices with Leica UC6 Ultramicrotome on a glass knife. The sections were contrasted and visualized at 80 kV using a TEM JEOL JEM-1010 at the Centre of Electron Microscopy of Babeș-Bolyai University, Cluj-Napoca. Images were obtained with Mega View III camera (Bloom and Fawcet [1]; Kuo [17]; Watt [18]).

Antimicrobial analysis

The disk diffusion method was employed for determination of the antimicrobial activity of the ethanol extracts. Briefly, a suspension of the tested microorganisms (0.1 mL, 10^8 cells/mL) was spread on the solid Luria Bertani medium. 6 mm sterile filter paper disks were impregnated with 40% ethanol extract of both type of extracts, corresponding to 130 mg/mL, respectively 800 mg/mL, and placed on inoculated plates. *Salmonella enteritidis* ATCC 13076, *Shigella sonnei* ATCC 9290, *Enterococcus faecalis* ATCC 51239, *Pseudomonas aeruginosa* ATCC 27853, *Proteus* spp., *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis*, *Candida albicans* and *Saccharomyces cerevisiae* were used for this test. A negative control containing only 40% ethanol was also placed on the plates together with standard antibiotic discs containing 10 U ampicillin, 120 U gentamycin, 30 U cephalotin and 1,000 U clotrimazole. Plates were incubated at 37ºC (bacteria) for 24 hours or at 25ºC (fungi and *B. subtilis*) for 48 hours, adapted from Radulovic [7].

Statistical analysis

Throughout the manuscript data are displayed as mean ±SD from at least three independent experiments. Statistical calculations were performed with Gnumeric Spreadsheet (Gnome Office Suite, Gnome Foundation, Cambridge, MA) and comparison between groups was performed by Student's t-test and values of $p<0.05$ were considered significant.

3. Results and Discussion

It is very difficult to establish the range of protective potentials a certain plant can have. The composition of extract and the type of an extract's administration are decisive to the outcome of an experiment. Thus, the present study focuses on the effects of *Lycopodium clavatum* and *Equisetum arvense* hydroalcoholic extracts on acetaminophen-induced liver damage mice model. These two plants were chosen because are used as medicinal plants with known traditional usage in hepatoprotection and antimicrobial activity (Oh [8]; Radulovic [7]; Canadanovic-Brunet [19]; Wang [9]; Sinha [20]; Kukric [21]).

Pathak [14;15] used for *in vivo* mice experiments 90% hydroalcoholic *Lycopodium* spores extract to analyse the protective potential against a carcinogenic (p-dimetyl amino azobenzene) in mice liver and spleen. Lycopodine was the protective compound found mostly in the *Lycopodium* spores. For the carcinogenic effect to emerge, the treatment was administered for 90-120 days, with favourable results. We also used mice in our *in vivo* experiments, because mice have similar to human dose-dependent response for acetaminophen intoxication effect. 400 mg/body weight acetaminophen induce toxic hepatitis in both man and mice (Jaeschke [22]).

GC-MS analysis

Almost all literature data revealed the composition of *Equisetum arvense*, both hydroalcoholic and aqueous extracts, data obtained by HPLC analysis (Milovanovic [6]). Our study reveals the GC-MS analysis of *Equisetum arvense* and *Lycopodium clavatum* composition.

The principal hepatoprotective compound of *Lycopodium clavatum* was found to be lycopodine (Pathak [14]), one of the most abundant alkaloid (Figure 1A) in our ethanolic extract (58.26%). A

GC-MS analysis was made also for other extracts where were used other solvents, besides ethanolic extract: dichloromethane extract, hexane extract, methanolic extract (data not shown). Other compounds found in *Lycopodium* extracts in a high percent are phytol and phenol (Table 1). Similar studies on *L. clavatum* or other *Lycopodium* species found a wider range of compounds: alkaloids (e.g. lycopodine), terpene (e.g. acyclic diterpene alcohol – phytol), fatty acids, carbohydrates, alcohols, phenols, etc. (Takayama [23]; Orhan [4; 24]; Halldorsdottir [25]).

Table 1. *Lycopodium clavatum* and *Equisetum arvense* ethanolic extract compounds obtained through GC-MS.

	No.	**Compounds**	**Area% of Total**
Lycopodium clavatum	1	2-methoxy-4-vinylphenol	1.51
	2	5-octadecene	0.58
	3	Phenol, 2,4-bis(1,1-dimethylethyl)	12.40
	4	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene; (beta-pinene)	3.60
	5	Hexadecanoic acid; (palmitic acid)	6.18
	6	9,12,15-octadecatrienoic acid, methyl ester; (linolenic acid, methyl ester)	0.99
	7	Phytol	12.05
	8	9,12,15-octadecatrienoic acid, ethyl ester; (linolenic acid, ethyl ester)	0.84
	9	9,12,15-octadecatrien-1-ol	3.54
	10	Lycopodine	**58.26**
		Total	**99.95**
Equisetum arvense	1	2-furancarboxaldehyde, 5-(hydroxymethyl); (5-(Hydroxymethyl)furfural)	5.96
	2	5-octadecene	0.97
	3	Phenol, 2,4-bis(1,1-dimethylethyl)	29.12
	4	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene; (beta-pinene)	8.24
	5	Hexadecanoic acid; (palmitic acid)	20.81
	6	9,12,15-octadecatrienoic acid, methyl ester; (linoleic acid, methyl ester)	3.75
	7	Phytol	9.88
	8	9-octadecenoic acid; (oleic acid)	1.88
	9	9,12-octadecadienoic acid; (linoleic acid)	2.33
	10	9,12,15-octadecatrienoic acid, ethyl ester; (linolenic acid, ethyl ester)	0.88
	11	9,12,15-octadecatrien-1-ol	16.13
		Total	**99.95**

Equisetum arvense ethanolic extract contains compounds from all classes of compounds: phenols, fatty acids, alcohols, esters, carbohydrates, terpenes (Figure 1A; Table 1). *E. arvense* extracts contains a phenol derivate found in high percent in dichloromethane extract (data not shown). Phytol appears in other extracts too, as it was found in previous studies (Robu and Milica [26]; Ardelean and Mohan [3]) with the highest percent in hexane (data not shown). We also found 5-(hydroxymethyl) furfural (5.96% in ethanolic extract), a highly toxic and cross-reactive compound (Husoy [27]; Rosatella [28]; van Putten [29]). Comparing to similar studies we found none of the specific alkaloids for *Equisetum* genus (Oh [8]; Radulovic [7]; Milovanovic [6]; Mimica-Dukic [30]).

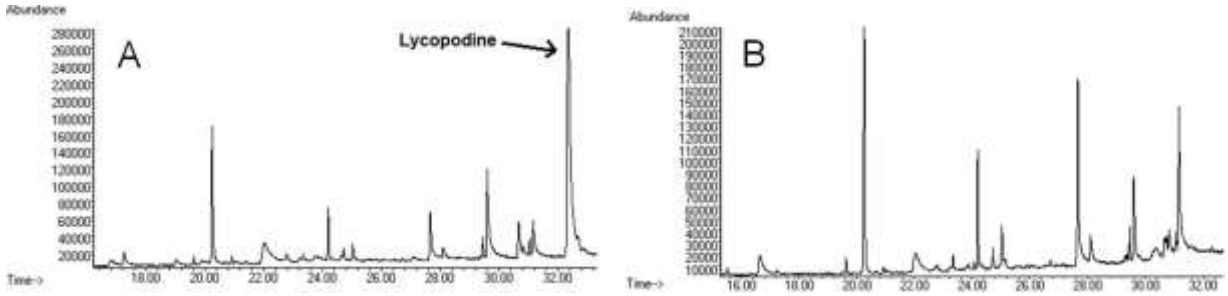

Figure 1. A - Chromatogram of *Lycopodium clavatum* ethanolic extract. B - Chromatogram of *Equisetum arvense* ethanolic extract.

AST and ALT analysis

Liver enzyme analysis showed low levels of transaminases for the absolute control group when compared to the acetaminophen treated group, sacrificed 4 days after injection. In the latter, AST units were four times higher, and ALT values doubled (Figure 2). The high level of transaminases translates to liver cellular death and lysis, the AST/ALT ratio indicates hepatitis, and AST values over 1000 U/L are interpreted as toxic hepatitis (Raurich [31]). The ethanol treated group had a significant ALT increase, and a low AST compared to control. The plant extract control groups had lower levels – close to the absolute control and a bit higher ones for the ALT (Figure 2). These results are consistent with previous studies (Baracho [32]).

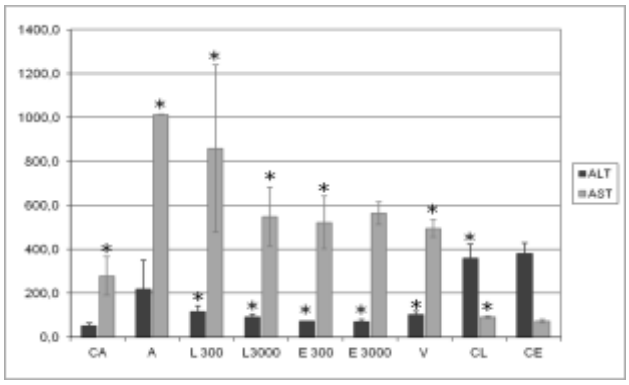

Figure 2. ALT-AST chart for mice intoxicated with acetaminophen and treated with *Lycopodium clavatum* and *Equisetum arvense* extracts.
* indicates significance between the relevant groups. Both transaminases are drastically increased in the acetaminophen group (A) compared to the untreated one (CA). Noticeable reductions appear between the four extract groups (L300-*Lycopodium* 300 mg/kg, L3000-*Lycopodium* 3,000 mg/kg, E300-*Equisetum* 300 mg/kg, E3000-*Equisetum* 3,000 mg/kg) and the acetaminophen group, respectively the vehicle group (V). The relative controls (CL-*Lycopodium* 300 mg/kg, CE-*Equisetum* 300 mg/kg) treated only with the ethanolic plant extracts indicate that the extracts alone do not induce hepatitis.

The acetaminophen-administered groups, treated with 300 mg dry extracts/kg body weight showed high levels of AST and a slightly lower one for ALT, compared to the acetaminophen intoxicated group. A noticeable decrease for both transaminases was seen in the groups treated with 3,000 mg dry plant extracts/kg of body weight (Figure 2). Even though there is a certain decrease of enzyme values compared to the acetaminophen-treated group or the ethanol-treated group, the AST/ALT ratio still indicates hepatitis (Figure 2).

Histological analysis

Baracho [32] studied the effects of *Equisetum arvense* water extracts in graded doses on normal rats and the histological results showed no significant morphological and hepatic function changes.

Liver of the untreated mice had the healthy, normal structure with large hepatocytes arranged in cords around a centrilobular vein, forming hexagonal shaped lobules, with interlobular veins, arteries and biliary ducts between lobules (Copstead and Banasik [33]) as shown in Figure 3A, where the Kiernan space can be identified. The liver of acetaminophen intoxicated mice had massive infiltration of lymphocytes in necrosis areas, mostly around the centrilobular veins, as shown in Figure 3B. It can also be noticed the dilated sinusoids and affected hepatocyte nuclei. In Figure 3C and 3D, it can be seen that *L. clavatum* extract in both concentrations did not improve the aspect of the tissue, moreover at the 3,000 mg/kg body weight concentration the entire liver tissue seemed to be invaded with lymphocytes probably due to a severe state of inflammation. The liver of intoxicated mice, treated with *E. arvense* showed a significant improvement in their structure, but there still was an exaggerated dilatation of the sinusoids (Figure 3 E and F). Control mice treated only with ethanol had an extensive accumulation of lipid vesicles in the hepatocytes cytoplasm and a few areas of necrosis with infiltrated lymphocytes. Control mice treated only with the plant extracts presented some lipid accumulation, sinusoid dilatation and lymphocytes in large numbers at the sinusoids level (data not shown).

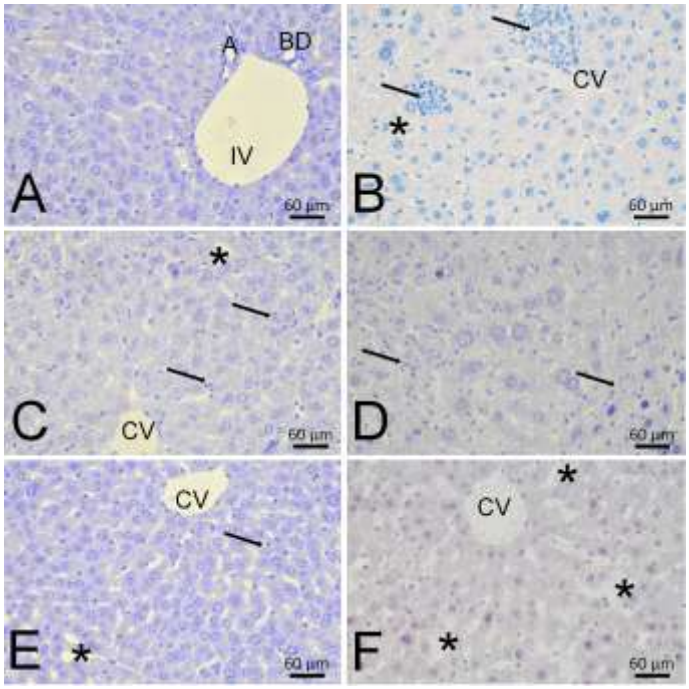

Figure 3. Histological images of mice liver intoxicated with acetaminophen and treated with *L. clavatum* and *E. arvense* ethanolic extracts.
A – Control mouse liver. The image shows the normal aspect of a Kiernan space with an interlobular vein (IV), interlobular artery (A) and biliary ducts (BD). **B** – Acetaminophen intoxicated mouse liver with areas of lymphocyte infiltration and necrosis (black arrow) and dilated sinusoids (asterisk) around a centrilobular vein. **C** – Acetaminophen intoxicated mouse liver treated with 300 mg/kg body weight *L. clavatum* extract. The liver presents lymphocyte infiltration (black arrow) and sinusoid dilatation (asterisk) near a centrilobular vein. D - Acetaminophen intoxicated mouse liver treated with 3,000 mg/kg body weight *L. clavatum* extract: the lymphocytes flood the tissue (black arrow). **E** - Acetaminophen intoxicated mouse liver treated with 300 mg/kg body weight *E. arvense* extract: some lymphocyte infiltration (black arrow) can be noticed and a general sinusoid dilatation (asterisk) in a large area around the centrilobular vein (CV). **F** - Acetaminophen intoxicated mouse liver treated with 3,000 mg/kg body weight *E. arvense* extract: extensive sinusoid dilatation (asterisk) in the area near the centrilobular vein (CV).

Immunohistological analysis

In order to see whether the liver inflammation was reduced by the plant extracts, we stained the liver samples for a number of inflammation markers: cyclooxygenase 1 and 2 (COX1 and 2), lipoxygenase 5, 12 and 15 (LOX5, 12, 15), ED1 (CD68) and ED2 (CD163).

Cyclooxygenases and lipoxygenases are enzymes that use arachidonic acid to form inflammation molecules such as prostaglandins and leukotrienes. COX1 is a constitutive enzyme in the liver, but its expression is enhanced together with that of COX2 in lesions, intoxications and liver affections (Wojcik [34]). In the control group the expression of COX1 (Figure 4) was weak but equally spread in the entire parenchyma, and it seems to be localized at the sinusoids level (endothelial cells). In the acetaminophen intoxicated liver, COX1 expression was perivasculary enhanced, as confirmed by other studies (Wojcik [34]). Liver of treated mice with plant extracts had an enhanced COX1 expression spread in the entire parenchyma and also focused to small groups of hepatocytes. Mice treated with *L. clavatum* extract seemed to be more affected than those treated with *E. arvense* extract. The same expression pattern can be noticed for COX2 and LOX5 (Figure 4). The control group who was administered only the vehicle (40% ethanol) presented similar expression pattern as the acetaminophen intoxicated liver for COX1, COX2 and LOX5. Liver of control mice treated with plant extracts were similar in antigen expression to the acetaminophen intoxicated mice and plant treated mice, but with a weaker intensity (data not shown).

LOX12 and LOX15 have no significant expression in control mice, and only a weak, but well enclosed expression around veins. In the *L. clavatum* and *E. arvense* treated mice, LOX12 and LOX15 appear at the sinusoids level, also with weak intensity (Figure 4). For the relative control groups no expression was noticed for LOX12 and LOX15 (data not shown). ED1 and ED2 are specific for monocytes and macrophages, which comprise almost 20% of the total number of cells in the liver (Bouwens [35]). This percent increases in inflammation when macrophages extravasate in the tissue to find and eliminate dying cells or infectious elements (Santos [36]). In control group, both ED1 and ED2 have a normal weak expression, dispersed throughout the tissue and localized to sinusoids. In acetaminophen intoxicated group the expression seemed to be concentrated around blood vessels, due to the fact that macrophages migrate to the place of injury, and the tissue around the blood vessels is most exposed to intoxicants (Ambrust and Ramadori [37]). In the liver of plant extract treated mice the markers' expression is elevated and concentrated to several groups of hepatocytes throughout the tissue. ED1 has an enhanced expression in the *L. clavatum* treated mice (Figure 4), and this is consistent with monocyte infusion, which means inflammation (Karlmark [38]). The relative control groups were intensely coloured for ED1 and ED2 at the sinusoids level and for the ethanol group at necrosis sites also (data not shown). The same expression for these markers, in the acetaminophen intoxicated liver tissue and plant extract treated mice, shows that these extracts affect the most metabolically active hepatocytes - cells from the middle area of the lobule (Ross [39]). As the images show, the liver seemed to have eliminated the acetaminophen threat, but the plant extracts do not help with this detoxification, instead they affect the weakened active cells. One structural organisation of the lobule arranges hepatocytes by their principal function: the ones near to the portal triad are specialised in division and are considered young hepatocytes, which then migrate to the middle, where the mature and metabolically active hepatocytes are located; these as well migrate towards the centrilobular vein, where the old hepatocytes locate.

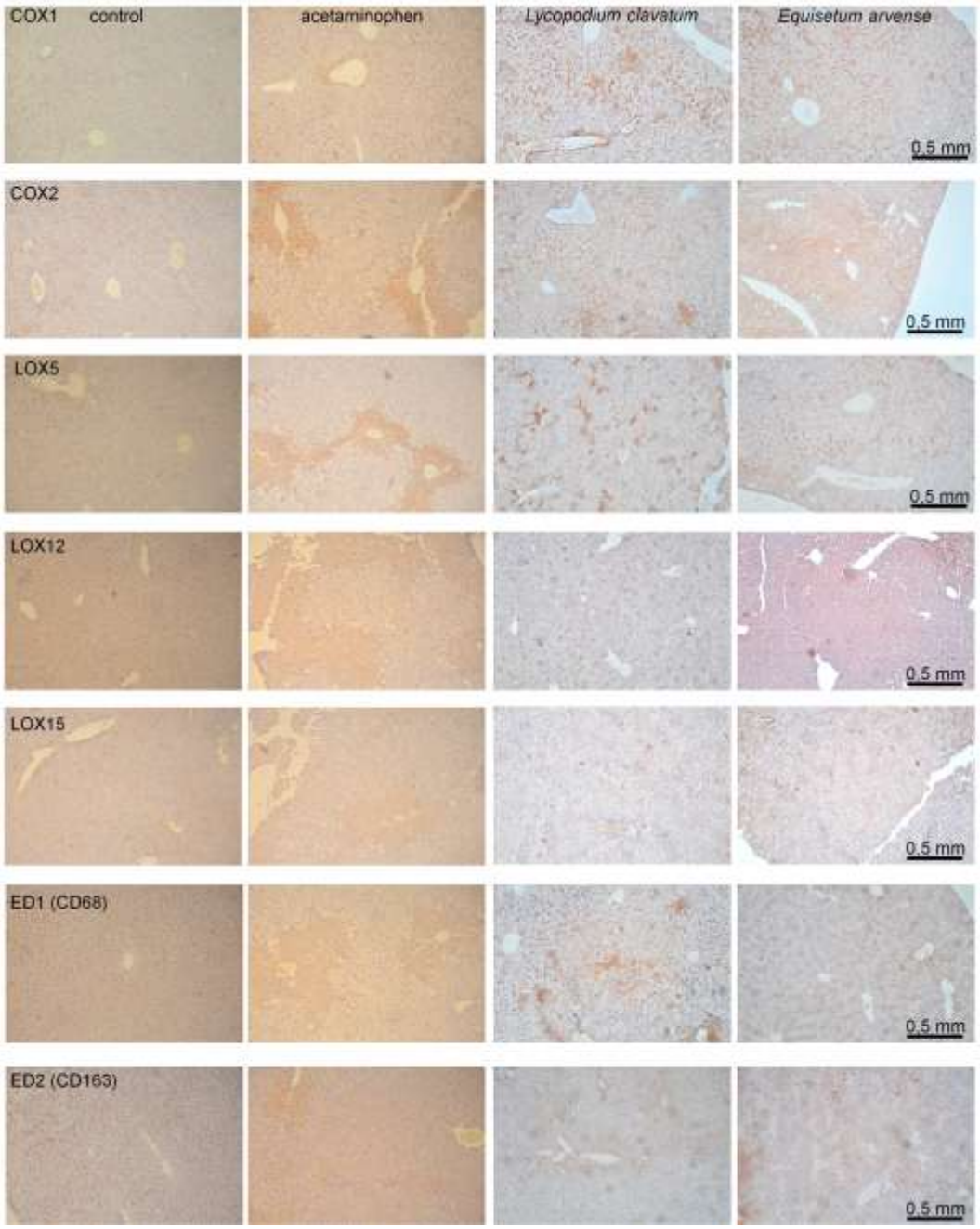

Figure 4. Immunohistological images* of mice liver intoxicated with acetaminophen and treated with ethanol extracts of *L. clavatum* and *E. arvense* 3,000 mg/kg body weight. *Specimens were stained for inflammation markers COX1, COX2, LOX5, LOX12, LOX15, ED1 (CD68) and ED2 (CD163). The images show an increased expression of markers around centrilobular and interlobular veins for the acetaminophen intoxicated mice in comparison to the control group. Acetaminophen induced liver injury mice treated with *L. clavatum* and *E. arvense* extracts showed a different expression pattern, the markers being spread in the parenchyma and not only in the perivascular areas.

This arrangement is needed specifically for protection from intoxicants, in which case the old cells, near the centrilobular vein, are the first to sacrifice (Ross [39]). This action of the plant

extracts, specifically *L. clavatum* extracts, has been observed in our laboratory in experiments involving highly metabolically active hepatocarcinoma cells and by others (Pathak [15]), which led us to believe that *L. clavatum* extract affects the mitochondrial activity.

Electron microscopy analysis

Control mice liver (Figure 5A) showed the classic structure of hepatocytes (Copstead and Banasik [33]). The acetaminophen-injured liver appears to have a reduced number of binucleate hepatocytes and an increased number of Kupffer cells, also hepatocytes have swollen, pinched and cristae-less mitochondria (Figure 5B). The canaliculi and sinusoids are also affected, appearing plane because of the missing and damaged microvilli.

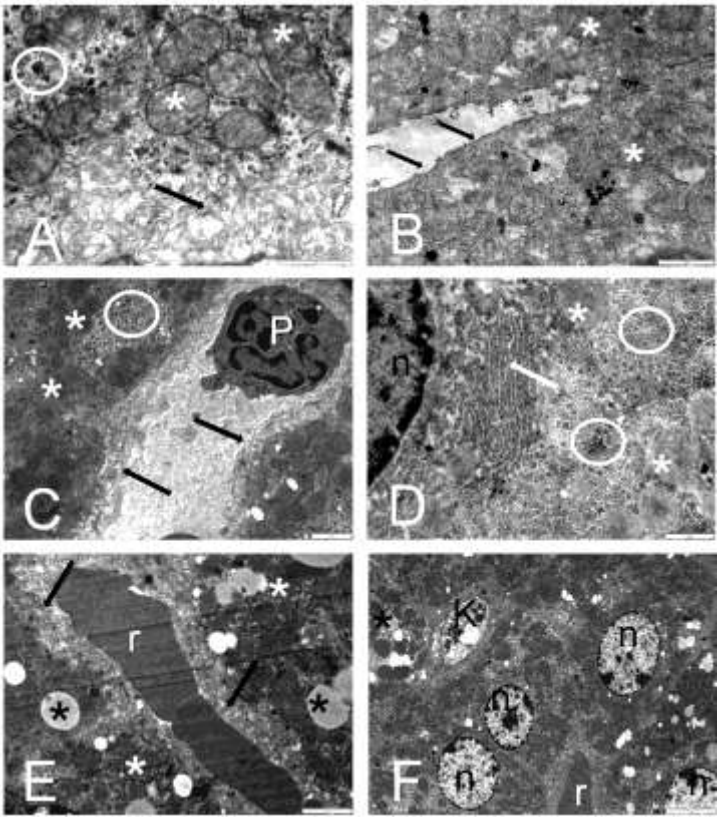

Figure 5. Electron micrographs of mice liver intoxicated with acetaminophen and treated with ethanol extracts of *Lycopodium clavatum* and *Equisetum arvense*
A – Liver of absolute control group (bar = 1 µm). The normal aspect of liver can be seen: large number of mitochondria (white asterisk), glycogen formations (white circle) and microvili in the bile canaliculi (black arrow). **B** – Liver of acetaminophen-intoxicated group. The sinusoid lacks the microvili (black arrow), and mitochondria are swollen (white asterisk), disintegrated and without cristae (bar = 2 µm). **C** – Liver of acetaminophen-intoxicated mouse that received 300 mg *L. clavatum*/kg body weight (bar = 2 µm). A circulating polymorphonuclear cell (P) appears in the micrograph, located in a sinusoid that is lined with microvilli (black arrow). Large areas with glycogen rosettes (white circle) are also present and mitochondria are surrounded by endoplasmic reticulum (white arrow). **D** – Liver of acetaminophen-intoxicated mouse that received 3,000 mg *L. clavatum*/kg body weight (bar = 1 µm). The image displays a round hepatocyte nucleus (n) close to a large area of rough endoplasmic reticulum (white arrow) and glycogen formations (white circles). The mitochondria are also numerous and cristae are visible (white asterisk). **E** – Liver of acetaminophen-intoxicated mouse that received 300 mg *E. arvense*/kg body weight. Although microvili (black arrow) are unaffected, red blood cells (r) fill up most of sinusoids and lipid droplets (black asterisks) are numerous (bar = 2 µm). **F** – Liver of acetaminophen-intoxicated mouse that received 3,000 mg *E. arvense*/kg body weight. A binucleated hepatocyte (n) having great number of mitochondria appears in the image, together with a Kupffer cell (K) and a sinusoid blocked by red blood cells (r). Few small lipid droplets (black asterisk) can also be noticed (bar = 5 µm).

The group of mice with acetaminophen-induced liver injury and treated with 300 mg *L. clavatum*/kg body weight (Figure 5C) have many small lipid droplets in their hepatic cells and many Kupffer cells seem to invade the tissue as a sign of inflammation, but the hepatocyte organelles (mitochondria, nuclei, endoplasmic reticulum) seem intact. The mice group treated

with 3,000 mg *L. clavatum*/kg body weight have shown a significant modification in their ultrastructure (Figure 5D), having many reduced areas of focal necrosis between areas of seemingly normal tissue. Even so, unaffected areas have large hepatic cells with 1-2 large nuclei, each one with two to four nucleoli, suggesting a high protein synthesis activity.
For the treated liver with *E. arvense* the hepatoprotective effect is moderate. When administered in a concentration of 300 mg/kg body weight the liver displays a certain degree of stasis, noticeable by the large number of red blood cells packed up in the small vessels (Figure 5E). Numerous macrophages can still be found in the tissue and many lipid droplets inside the hepatic cells. The mice group treated with 3,000 mg of *E. arvense*/kg of body weight (Figure 5F) has a reduced number of Kupffer cells in the tissue compared to the acetaminophen treated group and to the previous *Equisetum* treated group. The bile canaliculi were unaffected, the nuclei, round and the endoplasmic reticulum, both smooth and rough, were present in large amounts, together with many mitochondria and glycogen formations; hepatic stasis is still present. The relative control groups have the ultrastructural anatomy typical for alcohol intoxication (Raurich [31]; Iancu and Manov [40]). The acetaminophen-intoxicated and ethanol-treated group of mice had lipid droplets and affected mitochondria in the hepatocytes (data not shown).

Antimicrobial analysis

The antimicrobial analysis revealed antimicrobial effects with preponderance on gram-negative bacteria. The microbiological analyses revealed few clear bacteriostatic activity areas around the filter discs impregnated with plant extracts.

Table 2. Antimicrobial effects of *Lycopodium clavatum* and *Equisetum arvense* extracts on reference microorganisms compared to specific antibiotics.

	SPECIES	ANTIBIOTICS/ANTIFUNGAL				PLANT EXTRACTS				40% ETHANOL
						130 mg/mL		800 mg/mL		
		AMP 10U	GN 30U	KT 120U	KLT 1000U	*Lycopodium*	*Equisetum*	*Lycopodium*	*Equisetum*	
Gram +	***B. subtilis***	30.3±1.5	23.4±0.6	33.3±1.4	20.6±0.7	0	7.7±1.2	7.2±2.4	8.1±0.1	0
	E. faecalis	63.8±0.7	23.8±0.7	40.3±4.2	21.8±0.4	0	0	0	0	0
	S. aureus	26.5±1.5	20.7±0.6	37±1.1	19.7±0.8	0	0	0	0	0
Gram -	***S. enteritidis***	16.4±4.7	21.9±2.4	21.6±0.3	0	0	11.3±1	0	0	0
	S. sonnei	11.4±2.4	0	15.4±0.4	0	9.7±1.3	0	0	0	0
	E. coli	0	0	15.5±0.4	0	0	0	0	0	0
	K. pneumoniae	0	17.8±1	10±0.6	0	0	0	7.3±1.1	8.5±0.7	0
	P. aeruginosa	0	23.2±1	0	18.30.6	18.6±0.6	21.8±4.3	28.9±1	25.4±1	0
	Proteus spp.	13.1±7.3	20.9±1	23.7±0.3	0	0	0	0	0	0
Fungi	***C. albicans***	0	0	0	35.4±2.2	0	0	0	0	0
	S. cerevisiae	0	0	0	15.5±0.8	0	0	0	0	0

AMP–ampicillin, GN–gentamycin, KT–cephalotin, KLT–clotrimazole. Numerical values represent the mean diameter of inhibition area measured in millimeters, including the 6 mm diameter filter disc ±SD.

Uslu [41] tested some *Equisetum arvense* ethanol extracts of various concentrations on Gram positive and Gram negative bacteria by disc diffusion method, but the extract (no. 22) similar with ours had no inhibitory zones. Milovanović [6] obtained notable inhibitory areas around filter discs impregnated with similar hydroalcoholic *E. arvense* extracts as ours for bacterial and fungal species, including *E. coli* and *S. aureus* where we obtained no effect. Other reports (Sinha [20];

Kukric [21]) found that some gram negative and gram positive bacteria have little or no susceptibility to *E. arvense* ethanolic extracts.
The *E. arvense* ethanol extracts had a certain bacteriostatic effect against *Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Salmonella enteritidis*. The effect was stronger at a higher concentration of 800 mg/mL (Table 2).
L. clavatum extract expressed weak bacteriostatic effects against *Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Shigella sonnei*, stronger influence being exhibited at a concentration of 800 mg/mL (Table 2). The most affected strain was *P. aeruginosa,* as confirmed by other studies (Milovanovic [6]). Fungal species were not affected by the plant extracts. All microorganisms were inhibited by their specific and even unspecific antibiotic, respectively antifungal. The 40% ethanol alone had no effect against any of the tested species (Table 2).

4. Conclusions

In our experimental model were tested 40% ethanol (hydroalcoholic) extracts of two plants from spontaneous Romanian flora, *Lycopodium clavatum* and *Equisetum arvense,* against their hepatoprotective and antimicrobial activity on a mouse model. The extracts were analyzed by GC-MS, rarely used for these types of extracts, and important volatile compounds amongst others, as lycopodine from *Lycopodium clavatum,* and as a phenol from *Equisetum arvense* were found. These types of extracts were administered to acetaminophen induced liver injury in mice and the effects were monitored by several techniques: ALT-AST analysis, histological analysis, immunohistological analysis, electron microscopy analysis, antimicrobial activity.
For *Lycopodium clavatum* it was not observed any hepatoprotective activity at a concentration of 300 mg/kg of body weight, compared to a concentration of 3,000 mg/kg of body weight, which had a reduced hepatoprotective activity. In the case of hydroalcoholic extract of *Equisetum arvense,* a concentration of 300 mg/kg did not presented any significant hepatoprotective activity, and only a moderate one in a concentration of 3,000 mg/kg body weight extract. The reduced protective activity can be inferred from: the elevated levels of transaminases in the serum of mice treated with plant extracts compared to the acetaminophen-treated group; from the lack of improvement in liver structure and ultrastructure observed by several analyses: histological, immunohistological and electron microscopy.
The antimicrobial effects of the two plant extracts is also reduced to a moderate bacteriostatic effect when was used 130 mg/ml, and a significant bacteriostatic effect when was used the higher concentration of 800 mg/ml. None of the extracts gave a bactericidal effect comparable to the reference antibiotics.
To our knowledge there are no other studies addressing the compounds content, hepatoprotective and antimicrobial aspects of the Western Romanian spontaneous *Lycopodium clavatum* and *Equisetum arvense* species. By this study we have added new insights about these local plants with medicinal potential.

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