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Original Research

The influence of solvent nature on the extraction yield of *Pleurotus pulmonarius*, *Pleurotus floridanus* and *Pleurotus sajor-caju* and study of the effects of extract mixtures on oxidative stress

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Abstract

Oxidative stress plays an important role in the aetiology and pathogenesis of many chronic diseases. Edible mushrooms, especially *Pleurotus* spp., are considered to be a natural source of potential antioxidants.

This work investigated the influence of solvent nature on the extraction yield of *Pleurotus pulmonarius*, *Pleurotus floridanus* and *Pleurotus sajor-caju* and the influence of extract mixture concentrations on oxidative stress.

Extracts from each mushroom species and mixtures (1w:1w:1w) were produced by macerating the powders (1w:5v) in different solvents: distilled water, ethanol, hexane, ethyl acetate and dichloromethane.

The extraction yield was then determined and the antioxidant activity was evaluated for the extracts and formulations, with ascorbic acid used as a standard.

Based on the best yield and antioxidant activity *in vitro*, the aqueous extract mixture was used for acute toxicity testing (at 2000 mg/kg BW), and its effects at 250 and 500 mg/kg BW over 14 days on oxidative stress induced *in vivo* by lead acetate (35 mg/kg BW every two days) were compared. Ascorbic acid was used as a reference.

Oxidative stress markers were assessed in serum and homogenized tissue. We found that water had the best yield (66.32% for *P. pulmonarius*, 68.56% for *P. floridanus* and 65.40% for *P. sajor-caju*), followed by the ethanol, ethyl acetate, dichloromethane and hexane extracts.

In addition, all extracts and formulations had significant ferric reducing antioxidant power at C = 1 mg/ml (62.59–546.67 mg AAE/g), good scavenging activity as assessed by the DPPH free radical assay at C = 25 mg/ml (63.08–78.52%) and significant polyphenol content at C = 100 mg/ml (1.69–26.50 mg CE/g) with the best results seen with the aqueous extracts.

The aqueous extract mixture showed no particular signs of toxicity.

After lead exposure, significant increases were observed with respect to serum transaminase activity and creatinine and urea levels, but a decrease in total protein levels was found.

In addition, a significant increase in malondialdehyde levels but a decrease in reduced glutathione levels and superoxide dismutase, catalase and total antioxidant capacity was noted in the serum, liver and kidneys.

Treatment with the mixture brought about a significant amelioration in terms of antioxidant parameters, with the greatest effect seen with a dose of 500 mg/kg BW, as well as with the ascorbic acid used as a reference. The results here suggest that these mushrooms, due to their antioxidant potential, are beneficial for health and could be useful for the production of medicinal products and nutraceuticals.

Keywords: Extraction yield, antioxidant potential, oxidative stress, edible mushrooms, *Pleurotus spp*.

Introduction

Generally, oxygen is an indispensable molecule for life and the development and adaptability of aerobic organisms that primarily use it as a substrate within the respiratory chain for the production of ATP. Metabolism induces the production of reactive oxygen and nitrogen species in balance with antioxidant systems. However, excessive production of free radicals or a decreased antioxidant defence capacity can cause cellular damage encompassing disruption of intracellular oxidative status and the appearance of oxidative stress [1, 2].

Oxidative stress represents the inability of the body to defend itself against the aggression of reactive oxygen species due to the existence of an imbalance between the production of these species and the antioxidant capacity [3]. It plays a central role in the initiation, progression and malignancy of many cancers, and in the development of numerous pathologies such as cardiovascular disease, neurodegenerative diseases (such as Alzheimer's disease and Parkinson's disease), obesity, atherosclerosis, high blood pressure, diabetes and more [4].

To protect against oxidative stress, a balance between pro-oxidants and antioxidants must be maintained. To do this, the body has its own antioxidant system (formed from vitamins, enzymes and trace elements) that regulates the production of pro-oxidant molecules and prevents excessive cellular damage. However, the effectiveness of this system is reduced with the overproduction of pro-oxidant molecules [1]. Diet plays a very important role with respect to the intake of exogenous antioxidants that can support the endogenous antioxidant effect [5].

Nutrition is one of the main concerns of all societies in the world. Food supply should be both an economic and ecological subject. Health and nutrition involve the consumption of balanced and sufficient functional food components [6]. Edible mushrooms are filamentous fungi with fruiting bodies that demonstrate a huge number of pharmacological applications with respect to human health. They are considered to be delicious foods and are widely produced worldwide [7]. They are also considered to be a source of nutrition and provide pharmacologically important bioactive compounds that are useful in medicine. The consumption of mushrooms to help fulfil human nutritional requirements has been a common denominator throughout the history of humankind.

Their increased nutritional importance is due to the nutritive value of high-grade mushrooms, which almost equals that of milk. Mushrooms constitute an integral part of the normal human diet, mainly for their unique flavour, meaty taste and medicinal value, and are considered to be valuable health foods because of their low calories, fat and essential fatty acid content, and their high vegetable protein, vitamin and mineral content [8]. Moreover, mushrooms synthesize a range of secondary bioactive molecules (phenols, polysaccharides, pigments, tocopherols, terpenes and steroids) of high therapeutic value. These molecules have pharmacological properties such as antimicrobial, antiviral, anti-inflammatory, antitumour, antiallergic, antiageing, antidiabetic, anti-Alzheimer, hypocholesterolaemic and antioxidant activity. Among these metabolites, phenolic and flavonoid compounds show excellent antioxidant capacity. These metabolites can be extracted with water or different organic solvents [9].

Recent studies have shown the multipurpose applications of several mushrooms, including *Lentinula edodes* (Berk.) Pegler [10], *Pleurotus flabellatus* Sacc. [11], *Pleurotus* florida (Mont.) Singer [12], *Pleurotus ostreatus* (Jacq.) P. Kumm. [13], *Pleurotus sajor-caju* (Fr.) Singer [14] and *Ramaria aurea* (Schaeff.) Quél. [15], among others.

However, it has been observed that many investigations have been conducted on highly nutritious *Pleurotus* mushrooms, confirming their

medicinal importance [16]. *Pleurotus* species are found throughout the world and are among the most widely cultivated. In Cameroon, the most well-known edible mushrooms are those of the genus *Pleurotus*, of which the species *P. ostreatus* (whitish-greyish), *P. pulmonarius* (white), *P. sajor-caju* (beige-greyish), *P. floridanus* (beige), *P. citrinopileatus* (yellowish with white strips) and *P. salmoneo-stramineus* (pinkish) are the most cultivated, and popularized species are generally hybrids of the first four species.

For this study, three species, *P. pulmonarius*, *P. floridanus* and *P. sajor-caju*, have been chosen as samples because they are commonly consumed due to their availability, low cost of production, high product yield and easy cultivation procedures [17]. In addition, individually, they have shown significant antiradical, antihyperglycaemic and antidiabetic activity due to their levels of antioxidants and significant phenolic compounds [18].

Equally, Etoundi *et al*. showed that a formulation of the powders of these three species has remarkable antioxidant activity [19].

The focus of this work was the enhancement of species of edible mushrooms produced in Cameroon and their possible use as nutraceuticals since they demonstrate good antioxidant activity. To explore this, we investigated the influence of solvent nature on the extraction yield of *P. pulmonarius*, *P. floridanus* and *P. sajor-caju* and the influence of extract mixture concentration on oxidative stress.

Materials and methods

Sample collection, identification and processing

Samples of 3 edible mushrooms, *P. pulmonarius*, *P. floridanus* and *P. sajor-caju*, were harvested during the early mature fruiting stage (Nkolbong myciculture, Douala, Cameroon) during the dry season. Collected samples were identified based on their macro- and microscopic characterization following Kong [20] and Atri *et al*. [21]. Samples were individually cleaned and air-dried to dryness. They were then ground to a fine powder with the help of a grinder and stored for further study.

Extract preparation

Extraction was carried out with the aim of concentrating the bioactive compounds contained in the mushrooms in order to better evaluate the antioxidant activity. This was done by:

Maceration of powders

Powders of each of the 3 mushroom species were macerated for 48 hours in 5 different solvents: water, ethanol, hexane, ethyl acetate and dichloromethane, in a proportion of 1:5 (w/v). For each species, 50 g of powder were macerated in a volume of 250 ml of each solvent.

Filtration and evaporation

After 48 hours of maceration, the samples were filtered with filter paper and the collected filtrates were condensed using a rotary evaporator for ethanol, hexane, ethyl acetate and dichloromethane extracts and a vacuum pump for aqueous extracts. The extracts obtained after evaporation of the various solvents were stored in glass bottles covered with cotton to continue the complete evaporation at room temperature and to avoid contamination.

Once dry, the extracts were labelled, sealed and kept in a dry place for the preparation of different mixtures and antioxidant assays.

Determination of the extraction yield

Yield refers to the mass of the extract obtained after evaporation of the solvent; it is expressed as a percentage of the initial mass of the mushrooms (100 g), subject to extraction. It was calculated according to the following formula:

Y(%) = [Dry extract mass/Mushroom initial mass] × 100

Antioxidant activity of extracts

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined according to Etoundi *et al*. [19], with some modifications. The reaction mixture consisted of 100 µl of a solution of each extract at different concentrations (7, 10, 13, 15, 20 and 25 mg/ml) and 1 ml of 0.3 mM DPPH radical solution in ethanol.
Each test was repeated 3 times. After incubation for 30 min in the dark, at room temperature, absorbance was determined by a spectrophotometer at 517 nm. The results were presented as the DPPH radical scavenging percentage (% S), calculated according to the formula:

$$\% S = [(A_{control} - A_{sample})/A_{control}] \times 100$$

$A_{control}$ = Absorbance of negative control at the moment of solution preparation
A_{sample} = Absorbance of sample after 30 min

Determination of total phenolic content

The total phenolic compound content of the dry mushroom extracts was determined using the Folin-Ciocalteu procedure [22], using catechin as a standard. 30 µl of the sample at 100 mg/ml were added to 1 ml of 1 N Folin-Ciocalteu reagent diluted 10 times and mixed before incubation for 1 hour at room temperature. Each test was repeated 3 times. Absorbance was measured at 765 nm. The total phenolic compound content was expressed as mg catechin equivalent (CE) per g dry mushroom extract (mg CE/g).

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was conducted according to the procedure described by Benzie and Strain [23]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6).
It was freshly prepared and warmed at 37°C.
To 2 ml of the FRAP reagent, 75 µl of each extract at 1 mg/ml were then added.
The reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 593 nm. The results were expressed as mg ascorbic acid equivalent (AAE) per g dry mushroom extract (mg AAE/g).

Extract formulation preparation

The formulations of the different extracts obtained from the different solvent types (water, ethanol, ethyl acetate, dichloromethane and hexane) were prepared in 1w:1w:1w proportions. For 3 g of the extract formulation, 1 g of extract from each of the 3 species of mushroom studied was weighed and mixed in a beaker, to perform the different antioxidant assays. These formulations were prepared with the aim of optimizing the antioxidant capacity of the extracts and increasing their activity. The antioxidant activity was evaluated using the DPPH and FRAP assays and the polyphenol content was assessed using the Folin-Ciocalteu procedure as described earlier.

Evaluation of the toxicity of the aqueous extract mixture in rats

Acclimatization of subjects for each experiment

Mature male and female albino Wistar rats weighing 145–200 g obtained from the Animal Biology Department of the Faculty of Sciences of the University of Douala were used in this study. The animals were acclimatized for 2 weeks before the start of each experiment, in the Laboratory of Biochemistry of the Faculty of Sciences of the University of Douala, maintained under controlled temperature (23 ± 2°C) and humidity (50 ± 5%) conditions with a 12-hour light–dark cycle. The animals were housed in sanitized polypropylene cages covered with stainless steel mesh and containing a thick layer of sterile chips as bedding. The bedding of the cages was changed daily, while the cages were also cleaned daily. Animals had free access to standard laboratory rat pellets and water.

Ethics of experimentation

Animal experimentation was carried out in strict compliance with institutional and international best practice, with adherence to all relevant animal welfare guidelines.

Study of physiological and behavioural parameters

The protocol for the limit test proposed by the OECD (Organisation for Economic Co-operation and Development) [24] was used to assess the acute toxicity of the prepared extract mixture. This protocol recommends the administration of a single dose of 2000 mg/kg body weight (BW) of a substance to 1 experimental animal (rodent) followed by observation of the physiological effects on the animal for 48 hours. If the animal survives, 4 additional animals are treated and receive a dose of the substance at 2000 mg/kg BW.

The observation of the physiological effects on the animals in this case is carried out for 2 weeks.

Experimental design

Eight mature female albino Wistar rats were used in this experiment.

The rats were divided into 2 groups of 4 and treated only once by oral gavage using an orogastric tube with distilled water (1 ml) for the negative control group and with the aqueous extract mixture at a dose of 2000 mg/kg BW for the test group. Physiological and behavioural parameters were then observed for 2 weeks.

Organ assessment and assay of biochemical parameters of toxicity

At the end of the experiment, rats were deprived of food overnight for 12 hours and were euthanized by incision at the jugular vein after being anaesthetized with ketamine. Different organs were excised for macroscopic observation and blood was collected in dried test tubes. Serum was separated by centrifugation for toxicity marker analyses [aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, creatinine and urea].

Evaluation of the effects of the aqueous extract mixture concentration on oxidative stress *in vivo*

Experimental design

Twenty-four male albino Wistar rats were used in this experiment and allocated randomly to 6 groups of 4 rats each that were treated as follows:

Group 1: The reference group (RG) received deionized water 1 ml daily for 14 days.

Group 2: The negative control (NC) received deionized water 1 ml daily for 14 days and a solution of lead acetate 35 mg/kg BW every 2 days.

Groups 3 and **4**: Test groups (TG_{250} and TG_{500}) received the aqueous extract mixture at 250 and 500 mg/kg BW daily for 14 days and the lead acetate solution 35 mg/kg BW every 2 days.

Groups 5 and **6**: Positive controls (PC_{250} and PC_{500}) received an ascorbic acid solution 250 and 500 mg/kg BW for 14 days and the lead acetate solution 35 mg/kg BW every 2 days.

The various solutions were administered by oral gavage and animals within different treatment groups were maintained on a standard laboratory diet with free access to water.

Extracts were administered 4 hours after lead acetate administration. At the end of the experimental period, rats were deprived of food overnight for 12 hours and the animals were euthanized. The collected blood was used to obtain serum after centrifugation and stored at -80°C for further analysis.

The liver and the kidneys were excised, weighed and rinsed in saline. 1 g samples of fresh liver and kidney tissue were homogenized in Tris-HCl buffer (pH 7.4). Homogenates were centrifuged and the resulting supernatants were aliquoted and stored at -80°C for oxidative stress marker analysis.

Biochemical analysis

Total protein [25], creatinine [25], urea, ALT and AST [26] levels were determined in serum.

Determination of antioxidant levels and assessment of lipid peroxidation

The activity of superoxide dismutase (SOD) [27] and catalase (CAT) [28], as well as reduced glutathione (GSH) levels and the total antioxidant capacity (TAC) [23, 29], were assessed in serum and in the liver and kidney homogenates. Lipid peroxides in serum and the liver and kidney homogenates were measured using the TBARS assay [30].

Statistical analysis

All assays were carried out in triplicate and results are expressed as the mean ± standard deviation (SD). The data were inserted into an Excel spreadsheet (Microsoft Office 2013) and then analyzed using Statgraphics Centurion XV version 17.1.12 software. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test. Values of $p<0.05$ were considered significant.

Results

Extraction yields of different extracts

The extractions for the three species of mushroom from the five solvents resulted in 15 extracts: three aqueous, three ethanolic, three ethyl acetate, three dichloromethane and three hexane. The yields of the different extracts for each species of mushroom, obtained after extraction, are presented in **Table 1**. This table shows that for each species of mushroom, water performed better than all other solvents, followed by ethanol, ethyl acetate, dichloromethane and hexane. This could be due to the vast majority of compounds present in mushrooms being water-soluble.

Table 1 Extraction yields of the different extracts for each species of mushroom

Extraction solvent	Water	Ethanol	Ethyl acetate	Dichloromethane	Hexane
Species	**Extraction yield (%)**				
P. pulmonarius	66.32	49.91	49.60	44.36	43.44
P. floridanus	68.56	50.52	49.68	45.24	42.80
P. sajor-caju	65.40	55.91	49.72	44.89	42.40

Antioxidant activity of extracts

DPPH radical scavenging activity

The scavenging activity of the different extracts at various concentrations was evaluated using the DPPH assay. The free radical scavenging percentages obtained at the higher concentration of 25 mg/ml are presented in **Table 2**. This table shows that at a concentration of 25 mg/ml there was a significant difference between the inhibition percentages for the extracts of each species ($p<0.05$). For *P. pulmonarius* and *P. floridanus*, the aqueous extract (Eaq) had the highest scavenging percentage, followed by the ethanol extract (Ee), ethyl acetate extract (Eae), dichloromethane extract (Ed) and hexane extract (Eh).

For *P. sajor-caju*, the Ed and Eh values were higher than that for the Eae but these differences were not significant. However, no extract had a higher percentage of inhibition than that of vitamin C used as a reference.

Table 2 DPPH radical scavenging percentages for mushroom species extracts, compared to ascorbic acid

Extract	Eaq	Ee	Eae	Ed	Eh	AA
Species	**DPPH radical scavenging percentage (%)**					
P	68.21 ± 0.34^{b}	65.24 ± 0.10^{c}	65.19 ± 0.10^{c}	64.16 ± 0.10^{d}	63.65 ± 0.36^{d}	71.51 ± 0.71^{a}
F	69.29 ± 0.88^{b}	66.32 ± 0.30^{c}	65.75 ± 0.10^{c}	$64.90 \pm 0.20^{c,d}$	63.08 ± 0.20^{e}	
S	71.23 ± 0.36^{a}	68.89 ± 0.30^{b}	65.07 ± 0.20^{c}	65.98 ± 0.20^{c}	65.58 ± 0.10^{c}	

Eaq = aqueous extract; **Ee** = ethanol extract; **Eae** = ethyl acetate extract; **Ed** = dichloromethane extract; **Eh** = hexane extract; **P** = *P. pulmonarius*; **F** = *P. floridanus*; **S** = *P. sajor-caju*; **AA** = ascorbic acid; **C** = 25 mg/ml; **a, b, c, d, e** denote significant difference, $p<0.05$. Data are expressed as the mean ± SD of triplicate measurements

Total phenolic content of extracts

The Folin-Ciocalteu assay was used to determine the total polyphenol levels in the extracts expressed in mg CE/g, at a single concentration of 100 mg/ml.

The results are presented in **Table 3**. This table shows that there was a significant difference in the polyphenol content of the extracts of each species (p<0.05). For all species, the Eaq had the highest polyphenol levels followed by the Ee, Eae, Ed and Eh.

FRAP assay of extracts

The FRAP test determined the ability of the samples to reduce iron at low pH.

The iron-reducing power of the various extracts is expressed in mg AAE/g, at a single concentration of 1 mg/ml. The results are presented in **Table 4**.

These show that there was a significant difference in the FRAP of the extracts of each species (p<0.05). In addition, for all species of mushroom, the Eaq had the best FRAP results followed by the Ee, Eae, Ed and Eh.

Extract formulation preparation and evaluation of antioxidant activity

The extract formulations were established for each type of extract in proportions of 1:1:1. The result was five extract formulations: one formulation of aqueous extracts (Fmaq), one formulation of ethanol extracts (Fme), one formulation of dichloromethane extracts (Fmd), one formulation of ethyl acetate extracts (Fmae) and one formulation of hexane extracts (Fmh). Their antioxidant potential was evaluated using the FRAP, Folin-Ciocalteu and DPPH assays and the results are presented in **Fig. 1**.

This shows that for each antioxidant test there was a significant difference between the extract formulations (p<0.05).

In addition, the Fmaq had the greatest scavenging percentage (78.52 ± 0.26%), significantly higher (p<0.05) than vitamin C (71.51 ± 0.71%) used as a reference.

Similarly, it had the highest polyphenol content (26.50 ± 0.13 mg CE/g) compared to the other formulations, as well as the best FRAP result

Table 3 Total polyphenol content of mushroom species extracts

Extract	Eaq	Ee	Eae	Ed	Eh
Species	Total phenolic content (mg CE/g)				
P	16.56 ± 0.09^{b}	15.63 ± 0.08^{b}	9.63 ± 0.03^{d}	3.78 ± 0.01^{e}	1.78 ± 0.00^{f}
F	19.35 ± 0.07^{a}	15.35 ± 0.04^{b,c}	10.41 ± 0.05^{d}	3.82 ± 0.01^{e}	1.87 ± 0.00^{f}
S	18.76 ± 0.07^{a}	14.64 ± 0.04^{c}	9.82 ± 0.03^{d}	3.83 ± 0.01^{e}	1.69 ± 0.00^{f}

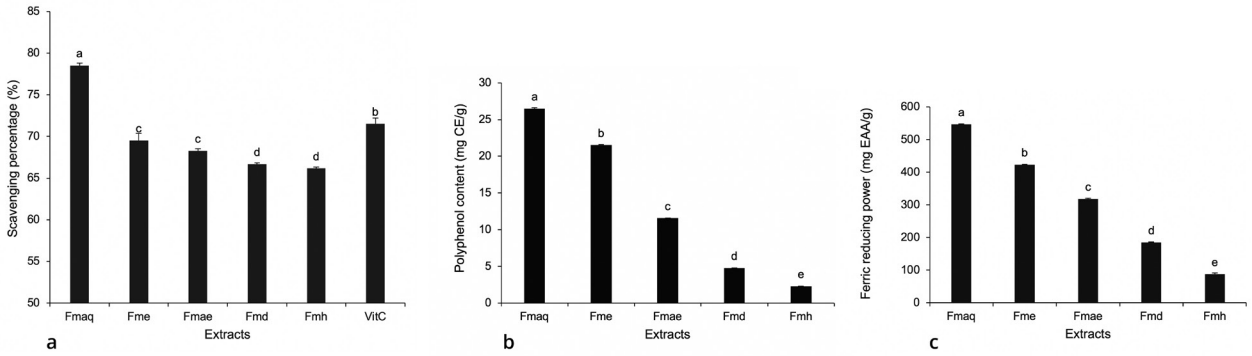
Eaq = aqueous extract; **Ee** = ethanol extract; **Eae** = ethyl acetate extract; **Ed** = dichloromethane extract; **Eh** = hexane extract; **P** = *P. pulmonarius*; **F** = *P. floridanus*; **S** = *P. sajor-caju*; **C** = 100 mg/ml; **a, b, c, d, e, f** denote significance, p<0.05. Data are expressed as the mean ± SD of triplicate measurements

Table 4 Ferric reducing antioxidant power of mushroom species extracts

Extract	Eaq	Ee	Eae	Ed	Eh
Species	Ferric reducing antioxidant power (mg AAE/g)				
P	531.85 ± 1.70^{a}	387.78 ± 3.31^{b}	294.44 ± 6.67^{c}	145.56 ± 6.94^{d}	75.93 ± 4.21^{e}
F	518.15 ± 2.3^{a}	400.37 ± 3.33^{b}	257.78 ± 4.44^{c}	153.33 ± 3.33^{d}	62.59 ± 3.57^{e}
S	524.81 ± 3.57^{a}	362.22 ± 5.56^{b}	232.78 ± 1.67^{c}	165.93 ± 4.21^{d}	64.81 ± 3.90^{e}

Eaq = aqueous extract; **Ee** = ethanol extract; **Eae** = ethyl acetate extract; **Ed** = dichloromethane extract; **Eh** = hexane extract; **P** = *P. pulmonarius*; **F** = *P. floridanus*; **S** = *P. sajor-caju*; **C** = 1 mg/ml; **a, b, c, d, e** denote significance, p<0.05.
Data are expressed as the mean ± SD of triplicate measurements

Figure 1 Antioxidant activity of extract formulations: **a)** DPPH assay, **b)** Folin-Ciocalteu assay, **c)** FRAP assay



Fmaq = aqueous extract formulation; **Fme** = ethanol extract formulation; **Fmae** = ethyl acetate extract formulation; **Fmd** = dichloromethane extract formulation; Fmh = hexane extract formulation; **VitC** = vitamin C; a, b, c, d, e denote significance, $p<0.05$. Data are expressed as the mean + SD of triplicate measurements

(546.67 ± 1.11 mg AAE/g). Thus, based on this assessment, Fmaq proved to be the best formulation and was consequently chosen for *in vivo* studies.

The Pearson correlation test was used to study the linear relationship between the different antioxidant tests.

The results are presented in **Table 5**.

This table shows that there was a strong, positive and significant ($p<0.05$) linear correlation between the scavenging activity, the FRAP results and the polyphenol content of the different extracts, for all species of fungus tested and their formulations.

Assessment of toxicity parameters related to the extract mixture in rats

Signs of toxicity

Toxicity indicators as observed in female rats after administration of 2000 mg/kg BW extract mixture after two weeks, compared to controls, are shown in **Table 6.**

No signs of toxicity in terms of either physiological or behavioural modifications were noted, and there were no deaths recorded.

In addition, macroscopic observation of the different organs (heart, liver, kidneys, lungs, spleen and pancreas) revealed no particular signs of inflammation in the female test subjects compared with the controls.

Table 5 Linear correlation between the different antioxidant tests: for extract formulations **(a)**; for *P. pulmonarius* (b); for *P. floridanus* **(c)**; for *P. sajor-caju* **(d)**

(**a**)	PC	SA	FRP
Polyphenol content (PC)		0.9722	0.9851
		(5)	(5)
		0.0278	**0.0022**
Scavenging activity (SA)	0.9722		0.9743
	(5)		(5)
	0.0278		**0.0257**
Ferric reducing power (FRP)	0.9851	0.9743	
	(5)	(5)	
	0.0022	**0.0257**	

(**b**)	PC	SA	FRP
Polyphenol content		0.9343	0.9950
		(5)	(5)
		0.0200	**0.0004**
Scavenging activity	0.9343		0.9643
	(5)		(5)
	0.0200		**0.0080**
Ferric reducing power	0.9950	0.9643	
	(5)	(5)	
	0.0004	**0.0080**	

(**c**)	PC	SA	FRP
Polyphenol content		0.9767	0.9783
		(5)	(5)
		0.0233	**0.0038**
Scavenging activity	0.9767		0.9171
	(5)		(5)
	0.0233		**0.0283**
Ferric reducing power	0.9783	0.9171	
	(5)	(5)	
	0.0038	**0.0283**	

(**d**)	PC	SA	FRP
Polyphenol content		0.9464	0.9795
		(5)	(5)
		0.0148	**0.0035**
Scavenging activity	0.9464		0.9760
	(5)		(5)
	0.0148		**0.0044**
Ferric reducing power	0.9795	0.9760	
	(5)	(5)	
	0.0035	**0.0044**	

(5) = Sample size (number of extracts per species); probability (in bold), $p<0.05$

Table 6 Changes in physiological and behavioural indicators

Toxicity signs	Control group	Test group
Locomotion	Normal	Normal
Reduced activity	-	-
Noise sensitivity	+	+
Breathing difficulties	-	-
Appetite	+	+
Coat	Normal	Normal
Urine	Normal	Normal
Stool appearance	Normal	Normal
Number of deaths	0	0

+ = yes; - = no; 0 = none. N=4 per group

Study of biochemical parameters relating to extract mixture toxicity

After observation of the organs and signs of toxicity in treated females that showed no particular evidence of injury, biochemical parameters related to toxicity were evaluated and the results are reported in **Table 7**.

There were no significant differences in AST, ALT, creatinine and total protein measurements between tested and control subjects.

Preventive effect of the Fmaq of the three species of mushroom on induced oxidative stress in rats

Effects of the extract mixture on serum biochemical parameters

As shown in **Table 8**, treatment with lead acetate (NC) caused a significant (p<0.05) increase in AST, ALT, creatinine and urea levels, when compared with the normal control group, while a significant decrease (p<0.05) was observed in total protein levels.

AST, ALT, creatinine and urea levels decreased significantly (p<0.05) in the TG and PC groups that received the extract

mixture and standard solution at two different concentrations, and there was an increase in total protein levels.

Table 7 Changes in biochemical parameters related to extract mixture toxicity

Group	ALT (IU/l)	AST (IU/l)	Total protein (g/l)	Creatinine (mg/dl)
Control	30.46 ± 0.89	36.28 ± 0.89	1.02 ± 0.03	6.63 ± 0.03
Test	29.29 ± 0.89	36.47 ± 1.21	1.00 ± 0.05	6.61 ± 0.04

Data represented as the mean ± SD. N=4 per group

Table 8 Ameliorative effects of the extract mixture on biochemical parameters after lead acetate exposure in rats

Group	AST (IU/l)	ALT (IU/l)	Protein (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)
RG	38.80 ± 0.89	42.49 ± 0.58	8.30 ± 0.22	0.96 ± 0.04	45.83 ± 7.22
NC	58.78 ± 0.58^{a}	61.50 ± 0.89^{a}	5.79 ± 0.29^{a}	1.20 ± 0.04^{a}	91.67 ± 7.22^{a}
TG_{250}	44.62 ± 0.34^{a,b}	51.99 ± 0.89^{b}	7.30 ± 0.07^{a,b}	1.05 ± 0.02^{b}	66.67 ± 7.22^{a,b}
TG_{500}	39.19 ± 0.34^{b}	45.20 ± 0.67^{b}	8.02 ± 0.23^{b}	1.00 ± 0.04^{b}	54.17 ± 7.22^{b}
PC_{250}	43.26 ± 0.89^{a,b}	53.93 ± 0.89^{b}	7.20 ± 0.27^{a,b}	1.04 ± 0.04^{b}	70.83 ± 7.22^{a,b}
PC_{500}	40.16 ± 0.58^{a,b}	44.81 ± 0.58^{b}	7.91 ± 0.23^{b}	1.01 ± 0.02^{b}	58.33 ± 7.22^{b}

Data are expressed as the mean ± SD (n=4) for each group. **RG** = reference group; **NC** = negative control; **TG_{250}** = test group at 250 mg/kg BW; **TG_{500}** = test group at 500 mg/kg BW; **PC_{250}** = positive control at 250 mg/kg BW; **PC_{500}** = positive control at 500 mg/kg BW. ^{a}Significantly different when compared to the reference group (p<0.05); ^{b}significantly different when compared to the negative control (p<0.05)

Thus, these results show a significant amelioration of the measured parameters, with superior amelioration seen with the 500 mg/kg BW extract mixture and standard solution dose.

Evaluation of antioxidant status indicators

Table 9 shows that in the serum, liver and kidneys from subjects within the NC group, lead acetate caused a significant (p<0.05) decrease in the following antioxidant parameters: CAT, SOD, GSH and TAC, when compared with data from the normal control group.

These parameters were significantly (p<0.05) enhanced in the TG and PC groups. In addition, levels of the lipid peroxidation biomarker, MDA, were significantly (p<0.05) increased in the NC group, compared to the RG.

This biomarker was significantly (p<0.05) decreased in the TG and PC groups.

Moreover, the TG_{500} and PC_{500} groups demonstrated a better ameliorative effect than the TG_{250} and PC_{250} groups.

Discussion

The evaluation of the antioxidant activity of the extracts and their formulations, using the DPPH, FRAP and Folin-Ciocalteu assays, showed that the Eaq for all the species and formulations had the highest activity in all the tests conducted, followed by the Ee, Eae, Ed and Eh. This could be explained by the difference in the extraction yields observed

Table 9 Effects of the extract mixture on serum, liver and kidney oxidative status indicators after lead acetate exposure in rats

Antioxidant parameter	Experimental group					
	RG	NC	TG_{250}	TG_{500}	PC_{250}	PC_{500}
Serum						
CAT (µmol H_2O_2/mg of prot)	98.33 ± 1.25	54.27 ± 0.76[a]	93.12 ± 1.38[a,b]	96.55 ± 1.08[b]	94.49 ± 0.97[a,b]	95.95 ± 1.13[b]
SOD (µmol/mg of	4.35 ± 0.11	2.68 ± 0.13[a]	3.54 ± 0.03[a,b]	3.86 ± 0.11[a,b]	3.58 ± 0.13[a,b]	3.92 ± 0.11[a,b]
GSH (µM)	34.31 ± 0.30	21.10 ± 0.29[a]	26.50 ± 0.11[a,b]	30.47 ± 0.37[a,b]	26.59 ± 0.22[a,b]	30.17 ± 0.30[a,b]
TAC (mmol/l)	2.29 ± 0.05	1.40 ± 0.03[a]	1.46 ± 0.03[a]	2.08 ± 0.06[a,b]	1.73 ± 0.05[a,b]	1.90 ± 0.07[a,b]
MDA (µM)	1.31 ± 0.07	2.42 ± 0.10[a]	1.87 ± 0.08[a,b]	1.49 ± 0.08[a,b]	1.90 ± 0.10[a,b]	1.65 ± 0.07[a,b]
Liver						
CAT (µmol H_2O_2/mg of prot)	118.87 ± 3.34	81.19 ± 0.93[a]	88.53 ± 0.70[a,b]	97.66 ± 0.62[a,b]	86.87 ± 0.81[a,b]	97.23 ± 0.64[a,b]
SOD (µmol/mg of	6.73 ± 0.03	4.99 ± 0.05[a]	5.83 ± 0.05[a,b]	6.39 ± 0.06[a,b]	5.85 ± 0.06[a,b]	6.40 ± 0.06[a,b]
GSH (µM)	77.67 ± 1.70	50.39 ± 0.48[a]	60.71 ± 0.56[a,b]	71.10 ± 1.10[a,b]	61.50 ± 0.70[a,b]	70.98 ± 0.99[a,b]
TAC (mmol/l)	3.81 ± 0.16	2.30 ± 0.06[a]	3.24 ± 0.02[a,b]	3.59 ± 0.04[a,b]	3.00 ± 0.09[a,b]	3.55 ± 0.08[a,b]
MDA (µM)	1.15 ± 0.02	2.79 ± 0.05[a]	2.35 ± 0.05[a,b]	1.79 ± 0.04[a,b]	2.36 ± 0.03[a,b]	1.77 ± 0.06[a,b]
Kidneys						
CAT (µmol H_2O_2/mg of prot)	115.09 ± 1.87	71.49 ± 0.80[a]	96.63 ± 0.89[a,b]	104.73 ± 1.00[a,b]	94.83 ± 0.54[a,b]	107.76 ± 0.55[a,b]
SOD (µmol/mg of	5.60 ± 0.07	3.16 ± 0.05[a]	4.46 ± 0.08[a,b]	5.35 ± 0.05[a,b]	4.48 ± 0.08[a,b]	5.51 ± 0.08[b]
GSH (µM)	73.33 ± 0.33	46.67 ± 0.19[a]	53.82 ± 0.53[a,b]	64.98 ± 0.56[a,b]	53.68 ± 0.60[a,b]	65.25 ± 0.35[a,b]
TAC (mmol/l)	2.29 ± 0.05	0.87 ± 0.05[a]	1.68 ± 0.06[a,b]	2.05 ± 0.04[a,b]	1.67 ± 0.04[a,b]	1.98 ± 0.02[a,b]
MDA (µM)	1.31 ± 0.07	2.38 ± 0.13[a]	1.80 ± 0.09[a,b]	1.60 ± 0.08[a,b]	1.85 ± .06[a,b]	1.50 ± 0.10[a,b]

Data are expressed as the mean ± SD (n=4) for each group. **RG** = reference group; **NC** = negative control; **TG_{250}** = test group at 250 mg/kg BW; **TG_{500}** = test group at 500 mg/kg BW; **PC_{250}** = positive control at 250 mg/kg BW; **PC_{500}** = positive control at 500 mg/kg BW. [a]Significantly different when compared to the reference group (p<0.05); [b]significantly different when compared to the negative control (p<0.05)

between the solvents used, namely water, ethanol, ethyl acetate, dichloromethane and hexane (**Table 1**), which would be influenced by their polarity. Indeed, as water is more polar than ethanol, ethyl acetate, dichloromethane and hexane (in descending order of polarity), it would have the capability to extract more (water-soluble) compounds than the others. Prabu and Kumuthakalavalli showed that for *P. floridanus* water performed better than ethanol [31]. In addition, edible mushrooms, being 95% water, would have more water-soluble compounds. This hypothesis is in line with that of Lee *et al*. who estimated that edible mushrooms of the genus *Pleurotus* would have more compounds that were soluble in water than in ethanol [32]. Moreover, for all types of extract, the formulations had better activities than single-mushroom extracts; better DPPH radical scavenging activity, better FRAP results and higher polyphenol levels. In addition, the Fmaq had the highest antioxidant activity. These findings corroborate the results of Etoundi *et al*., which showed that water extracts from established formulations had the best antiradical activity and the highest polyphenol content [19]. This could be due to the strong, positive, linear correlation between scavenging activity, FRAP results and the polyphenol content of extracts; that is, the increase in the scavenging activity of an extract and its FRAP are directly due to the presence of large amounts of phenolic compounds such as flavonoids, phenolic acids and tannins.

These have been described as natural antioxidants that are commonly found in mushrooms, with redox properties, and act as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers [33]. This was supported by the Pearson test **(Table 5)** which showed a strong, positive and significant correlation (p<0.05) between antioxidant tests for the extracts from all mushroom species studied and for their formulations.

Similarly, Etoundi *et al*. showed that there is a highly significant positive and linear correlation (p<0.001) between the DPPH radical scavenging percentages and the polyphenol levels of Eaqs from the formulations of the three edible mushrooms studied, with a correlation coefficient of $R^2 = 0.9063$ [19].

Moreover, in general, antioxidant power is strongly dependent on the concentration of phenolic compounds [34]. Our results corroborate those of Trabelsi *et al*. [35] who showed a significant and positive correlation between phenolic compound levels and antiradical activity.

The acute toxicity of the Fmaq of the three species of mushroom studied was determined according to the OECD protocol published in 2008. The results showed no particular signs of toxicity related to the extract mixture. This result corroborates those previously obtained by Etoundi *et al*. who assessed the toxicity of a formulation of *P. pulmonarius*, *P. floridanus* and *P. sajor-caju* powders and showed that it was not toxic. Similarly, Djomene *et al*. reported that edible *Pleurotus* mushrooms demonstrated no signs of toxicity. Thus, this study shows that with a mixture of Eaqs the reduced toxicity of these species of mushroom is retained [17, 36].

The oxidative stress induced in rats by administration of lead acetate resulted in a significant increase in serum levels of transaminases, creatinine and urea while a decrease in the total protein content was observed. These results agree with those of Offor *et al*. and Etoundi *et al*., which showed a considerable rise in transaminase, creatinine and urea levels and a reduction in the total protein content in response to a dose of 60 mg/kg BW and 35 mg/kg BW lead acetate, respectively [36, 37].

These results could be due to the binding of lead acetate to plasma proteins, where it causes alterations to a large number of enzymes. Thus, once in the body, it induces

liver dysfunction followed by high levels of serum enzymes, indicating leakage of aminotransferases from the hepatocyte cytosol into the bloodstream and loss of integrity of cell membranes. It can also disrupt protein synthesis in hepatocytes, thereby directing the metabolic activity towards the production of defence systems and gluconeogenesis. Degradation of liver proteins followed by the loss of catalytic function or structural stability could explain the increase in serum urea and creatinine levels in treated rats where proteins may be degraded to amino acids and then to urea and creatinine by the action of serum aminotransferases [38, 39].

However, the administration of the extract mixture had a protective effect here as demonstrated by the considerable reduction in the concentrations of creatinine, urea and transaminases in the blood and the increase in total protein levels. This indicates stabilization of the plasma membrane and regulation of liver function, which could be due to the reduction of bonds between toxic metabolites and the polypeptide chains of the proteins affected due to the administered extract. Indeed, the exceptionally high protein and essential amino acid content of fungi is of major dietary importance. The genus *Pleurotus* appears to be a source of the highest quality protein and several varieties have a good distribution of essential and non-essential amino acids [40, 41]. The presence of these amino acids in large quantities in fungi confers a protective effect in the face of oxidative stress through the restoration of the catalytic and structural integrity of proteins, as well as the regulation of their synthesis and cellular functioning [37].

Furthermore, the administration of a toxic dose of lead acetate resulted in a significant decrease in antioxidant enzymes (SOD and CAT), GSH and TAC, but a significant increase in MDA, in the serum, liver and kidneys of exposed rats. These results, corroborating those of Offor *et al*. and Etoundi *et al*. could be due to the fact that, in addition to targeting sulfhydryl groups, lead can also replace ions such as iron, copper, zinc or manganese, which serve as important cofactors for antioxidant enzymes, thereby leading to their inactivation. Another indicator of oxidative stress results from the actions of ROS on lipid membranes. The generated free radicals capture the electrons of lipids within cell membranes and damage the cells [36, 37].

A significant decrease in GSH levels could be explained by its participation in the elimination of the various toxic metabolites resulting from the degradation of xenobiotics such as lipid peroxides and electrophiles responsible for tissue damage [42].

The protective effects of the extract mixture compared to ascorbic acid on oxidative stress was subsequently assessed and the results obtained showed that this administration had a positive impact on biological markers of stress in the serum, liver and kidneys with an increase in TAC, GSH, CAT and SOD, and a decrease in MDA. Such effects reflect the antioxidant capacity of mushrooms, which is generally attributed to their abundance of phenolic compounds.

This protection has been ascribed to the genus *Pleurotus* in particular. Indeed, the role played by mushroom bioactive compounds in liver and kidney function has been the focus of considerable research interest for several years. It has been found that these compounds can repair liver damage due to toxins, protect against toxic agents, regenerate damaged hepatocytes, reduce inflammation and more [43].

Kozarski *et al*. showed that edible mushrooms contain all B vitamins, along with vitamins A, C, D, E, minerals and macronutrients, which are most necessary to the body [44]. These vitamins can chelate lead from tissues with restoration of pro-oxidant/antioxidant balance. Vitamin B6 (pyridoxine) and vitamin B1 (thiamin) have essential characteristics that

can prevent or reverse the deleterious effects of lead toxicity. Vitamin B6 is an important cofactor that participates in the metabolic trans-sulphuration pathway that is responsible for the synthesis of cysteine from dietary methionine. It also acts as an antioxidant by stimulating GSH production and is a moderate chelator [45]. The chelation of lead by vitamin B6 could be due to the presence of a nitrogen atom in its ring structure or the vitamin could interfere with the absorption of lead. Vitamin B1 has also been reported to have a protective effect against the short-term consequences of lead poisoning [46].

Similarly, the presence of minerals in this extract mixture could help restore the catalytic activities of the various antioxidant enzymes, as these include trace elements and transition metals essential in the defence against oxidative stress, acting as cofactors in enzyme reactions to maintain catalytic activity [47].

Thus, based on the results obtained after the daily administration of the extract mixture, we can confirm its protective effects against oxidative stress at a dose of 250 mg/kg BW, with an increased effect at 500 mg/kg BW. This was achieved through the combination of extracts from the different species of mushroom studied, which ensured a high concentration of bioactive compounds.

In addition, the administration of ascorbic acid doses equivalent to those of the extract showed the same effects on the parameters studied. Ascorbic acid traps oxygen free radicals that are by-products of many metabolic processes leading to oxidative stress [48].

It is probably the most widely studied vitamin for the prevention of lead-induced oxidative stress. Thus, its ability to extinguish ROS with metal chelation makes it a potential detoxifying agent for lead [49, 50]. An earlier study by Chang *et al*. showed the defensive actions of ascorbic acid on oxidative stress, which developed in the hippocampus of suckling rat pups exposed to lead. These authors reported that the introduction of ascorbic acid during pregnancy and lactation caused to some extent an improvement in oxidative stress in the developing hippocampus [51].

Conclusions

This study shows that the type of solvent used has a significant influence on the extraction yield of the edible mushrooms studied here. For all species, water performs best compared to other solvents. This testifies to the major presence of water-soluble compounds in mushrooms. In addition, all antioxidant tests (DPPH, FRAP, Folin-Ciocalteu) showed that water extracts from each of the mushroom species had better activities. Moreover, the extract formulations for these three species demonstrated an optimized antioxidant activity through their remarkable DPPH radical scavenging activity, their notable phenolic compound content and their relatively high FRAP, due to combination of the species. Thus, the extract formulations had better activities compared to the individual extracts of each species. The Pearson correlation test showed a positive and significant linear relationship between these different tests and this correlation can be attributed to the presence of large amounts of bioactive compounds (polyphenols, in particular) with antioxidant properties in mushrooms.

In vivo results showed that exposure to lead could generate oxidative stress, resulting in the elevation of transaminases and creatinine in serum, and a decrease in the total protein level. Lipid peroxidation in the liver and kidneys associated with the reduction in antioxidant status was also observed. Treatment with the aqueous extract mixture of the three types of mushroom studied here resulted in the prevention of lead-induced hepatorenal damage due to its antioxidant properties.

Thus, the aqueous extract mixture that had the strongest antioxidant potential could be suitable for use in the production of food and health-enhancing nutraceuticals. Regular consumption of these mushrooms, in combination, would confer greater efficacy with respect to the prevention of pathologies related to oxidative stress.

Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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