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Research Article

Acute toxicity and anti-inflammatory activity of an improved traditional medicine developed in Burkina Faso

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Abstract

An Improved Traditional Medicine (ITM) in capsule form presented by traditional health practitioners in Burkina Faso as used for hepatoprotective and hepatocurative treatment for viral hepatitis B. The study's main objective was to investigate the anti-inflammatory properties of this ITM, which is used in treating viral hepatitis B in traditional medicine in Burkina Faso. Phytochemical screening revealed the presence of flavonoids, saponins, tannins, coumarins, alkaloids, sterols and triterpenes. The *in vitro* anti-inflammatory assay inhibited LOX by 15.69 ± 0.50% at a 100 µg/mL concentration. The LD_{50} was estimated to be greater than 5000 mg/kg. *In vivo* activity using the carrageenan anti-oedema test showed good dose-dependent anti-inflammatory activity of ITM at 30, 65 and 130 mg/kg bw. After 3 hours, the extract at various doses reduced edema to over 50% inhibition. After 5 h, oedema was reduced to 85.209% at the 130 mg/kg. However, the reference acetylsalicylic acid (ASA) at 100 mg/kg bw showed a percentage of 85.048% at 5 h. The IC_{50} obtained after the ABTS, FRAP and LPO antioxidant tests of the phytomedicine were 351.00 µg/mL, 353.20 µM EAA/L and 81.13%. However, the IC_{50} with the DPPH method was higher than 10^4 µg/mL. The phytomedicine would have anti-inflammatory and antioxidant properties due to the presence of bioactive phytochemicals on inflammation and free radicals.

Keywords: ITM, Phytochemical, Acute toxicity, Anti-inflammatory, Antioxidants

INTRODUCTION

Hepatitis designates any inflammatory process in the liver [1]. The body's immune defense system destroys the infected cells, causing liver inflammation. The destruction of the virus is accompanied by massive consumption of oxygen, generating free radicals which, thanks to their unpaired electrons, attack the lipid and protein components of the cells and cause cell death [2]. If chronic, this process progresses to cirrhosis and hepatocellular carcinoma. Under physiological conditions, the free radicals produced are neutralised immediately by enzymatic and non-enzymatic antioxidants. This balance is upset in pathology because the defense mechanisms are insufficient to neutralise the excess free radicals [3]. There are several types of hepatitis, including viral hepatitis B. Viral hepatitis B is an infectious disease caused by the hepatitis B virus, commonly known as HBV. It results from an immune response secondary to the expression of HBV antigens on the surface of hepatocytes. Hepatocyte inflammation leads to the secretion of cytokines and chemokines and the lysis of infected cells, which accounts for much of the liver damage [4]. These inflammatory mediators act via

inflammatory cells active at the site of infection and release more reactive species [5].

Inflammation is the body's defence response to various aggressions that may be physical, chemical, biological (immune response) or infectious. These aggressive factors trigger a response from the immune system, the aim of which is to sound the alarm and recruit natural immune cells. This mechanism is crucial in the fight against infection, as it enables the phagocytosis of bacteria and foreign bodies [6,7]. Inflammation is mainly divided into acute and chronic inflammation, depending on different inflammatory processes and cellular mechanisms. Recent research has shown that inflammation is a major factor in the progression of several chronic diseases and/or disorders, including diabetes, cancer, cardiovascular disease, eye disease, arthritis, obesity, autoimmune diseases and inflammatory bowel disease [5,8]. Inflammation is also a major source of oxygen radicals produced directly by activated phagocytic cells, which are the site of a phenomenon known as the oxidative explosion, activating the NADPH oxidase complex.

Human cells are permanently exposed to reactive oxygen species (ROS), including superoxide or hydroxyl radicals. Oxidative stress is a negative effect produced by free radicals in the body. It has been the cause and pathogenesis of several diseases, such as viral infections, autoimmune pathologies, digestive system disorders, and neurodegenerative diseases [9]. Antioxidants form part of protective mechanisms in human cells to scavenge and neutralize these oxidants [10,11]. Moreover, oxidative stress leads to inflammatory processes through the release of cytokines and activation of enzymes such as lipoxygenases (LOXs) from inflammatory cells, which are involved in multiple physiological and pathological processes, including inflammatory and immune response, cell survival and apoptosis [12–14]. Consequently, these diseased conditions can be effectively alleviated by antioxidant compounds that neutralize free radicals [15].

Medicinal plants have been shown to scavenge reactive oxygen species and prevent lipid peroxidation since they possess natural antioxidants [16,17]. This is why more and more people are turning to traditional medicine for their healthcare. Various parts of plants have been used as medicine for many years [18,19]. Medicinal plants play a very important role in combating most diseases in sub-Saharan Africa because they are the source of many traditional medicines and contain phytochemical compounds that give them certain therapeutic properties and provide the raw material for modern medicine [20–22]. For example, *Cochlospermum tinctorium* (Bixaceae) and *Cassia sieberiana* (Fabaceae), the constituent plants of the ITM, are among the species considered to be the most effective against liver disease by traditional health practitioners in Burkina Faso [23].

Literature reported the anti-inflammatory, antioxidant, hepato-protective and hypolipidemic properties of several species [24–26]. Many species have Hepato-protective benefits and hypolipidemic activity [27–29]. The development of traditional medicine is now imperative for cultural, economic, and public health. Given the tissue damage caused by free radicals and the molecular mechanisms of inflammation on liver diseases, especially viral hepatitis (A, B, etc.), the use of plant-derived products could be an alternative, provided that they reduce the effects of inflammation and protect the liver from free radical damage. This is the context of our work, which involved assessing ITM's anti-inflammatory and antioxidant properties, developed by a traditional health practitioner, formulated in capsule form and used in traditional medicine to treat viral hepatitis B in Burkina Faso.

MATERIALS AND METHODS

Experimental plant products and animals

It consisted of the plant drug contained in the ITM capsules (combination of *Cassia sieberiana* DC (Fabaceae) and *Cochlospermum tinctorium* Perrier ex A. Rich (Bixaceae)). This ITM are formulated in capsules presented as hepatoprotective and hepatocurative in treating viral hepatitis B by traditional health practitioners in Burkina Faso. The capsules are packaged in boxes of 60 capsules with an average weight of 327 ± 8.58 mg, and the dosage is one (01) capsule per day to be taken for 06 months of treatment. The doses to be administered were based on this unit intake per day.

NMRI mice were used. These animals were provided by the Animal Facility of the Department of Medicine, Traditional Pharmacopoeia and Pharmacy (MEPHATRA/PH) of the IRSS.

Reagents

The following reagents were used: Neu reagent, PBS phosphate buffer, 0.67% 2-thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), iron dichloride ($FeCl_2$), iron trichloride ($FeCl_3$), aluminium trichloride ($AlCl_3$), sodium carbonate (Na_2CO_3), sodium nitrate ($NaNO_3$), Sodium chloride (NaCl 5% and 9%), Potassium chloride (KCl) Potassium chloride (KCl), sodium monohydrogen phosphate dodecahydrate (Na_2HPO_4, $12H_2O$), potassium dihydrogen phosphate (KH_2PO_4), 2,2'-azino bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulphate ($K_2S_2O_8$), Folin-Ciocalteu reagent (FCR) 2 N, ketamine, sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), potassium hexacyanoferrate 1%, sulphuric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipoxygenase 1-B, carrageenan (Sigma-Aldrich).

Determination of residual moisture content (RMC)

The residual moisture content of the plant material was determined by a thermogravimetric method based on the loss of water from the plant material during drying [30]. It was determined using the Halogen AHAUS MB35 desiccator. Three (03) test samples of ITM powder with a mass greater than 500 mg were weighed in triplicate and placed in a previously dried and tared watch glass. The whole was placed in the desiccator at 105 °C for ten (10) minutes [31]. At the end of the ten minutes, the RMC value was read directly from the instrument and displayed on the screen.

Phytochemical screening

The aim of the phytochemical screening was to determine the groups of bioactive chemicals present in ITM. The characterization tests of the different groups of substances were carried out in a liquid medium with chemical reagents specific to each group of bioactive substances. The method used was described by Ciulei and adapted by the Chemical Laboratory of the Research Institute for Health Sciences (IRSS) [32]. The principle of this method is based on the ability of the functional chemical groups of bioactive substances to react with specific chemical reagents to give their characteristic-coloured products. The $FeCl_3$ test has identified tannins (polyphenols); flavonoids by the Shibata reaction or cyanidin test; saponins by the foam test; steroids and triterpenes by the Liebermann-Burchard test; alkaloids by the Mayer and Dragendorff tests; Anthracenosides by Bornträger's test; coumarins and derivatives by Feigl's test.

The theoretical principles of high-performance thin-layer chromatography (HPTLC) are the basic principles of chromatography: separation of compounds based on their interaction with two immiscible phases (stationary and mobile phase). This screening method aimed to characterize the phytochemical groups present in ITM. Phytochemical groups such as phenolics, alkaloids, terpenes and steroids were identified using thin layer chromatography (TLC).

Phytochemical screening of extracts is carried out on HPTLC plates (10 cm × 10 cm) with silica gel 60 (Merck, Darmstadt, Germany). Approximately 10 µL of ITM extract and 3 µL of each standard are applied in an 8 mm strip along the 8 mm baseline from the bottom of the plate using a semi-automatic sample dispenser. The distance between the spots is 3.4 mm. The distance between the plate's first spot and left edge and between the plate's last spot and right edge is 20 mm. After coating, the plate is placed in a vessel containing the eluent (20 × 10 cm, saturation time: 30 minutes). Sterols, triterpenes, flavonoids and tannins were detected according to the methods described by H. Wagner and Blatt (1996) [33]. Depending on the metabolite to be identified, a specific solvent system was used for elution. A defined reagent was then used for each metabolite, and the plate was observed.

Total phenolic content

In alkaline medium, total phenolic compounds react with Folin Ciocalteu Reagent (FCR). The loss of a phenolic proton in an alkaline medium result in a phenolate anion capable of reducing the FCR in which the molybdate is reduced. This forms a coloured blue complex of molybdenum oxide with an absorption maximum of 760 nm. The intensity of the blue colour is proportional to the amount of total phenolics present in the sample [34].

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Total flavonoid contents

Aluminium chloride reacts with the oxygen atoms on carbon atoms 4 and 5 of flavonoids to form yellowish compounds. Flavonoids were determined by the method of Kumaran et al, adapted by Abdel-Hameed [35,36]. A volume of 2 mL of extract with 1 mg/mL concentration in methanol was mixed with 2 mL of aluminium trichloride ($AlCl_3$ 2%) in methanol. After 40 min, the absorbance was measured at 415 nm using a spectrophotometer (Agilent 8453). The white control tube consisted of 2 mL methanol. The absorbance of quercetin, used as a reference compound, was measured under the same conditions and used to obtain the correlation coefficient (R^2 = 0.9994) of the calibration curve. The assays were performed in triplicate. The amount of flavonoids in ITM in quercetin equivalent (QE)/g of extract was determined.

2.5. Acute Toxicity Assay

Toxicity testing was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 423 for acute oral toxicity [37]. Prior to the administration of the ITM extracts, females were divided into batches of 3 per cage and fasted for 4 h without food but with free access to water. For each extract, the test was conducted in two phases. In the first phase, 3 rats received a single oral dose of 2000 mg/kg bw of each extract. After administration of the extracts, the animals were observed every 15 min for 2 h, after which water and food were re-introduced. The mice were observed daily for up to 14 days post-treatment for potential signs of toxicity, such as behavioural or physiological changes, death and latency to death [38]. All animals were weighed before the start of the study and again at D1, D2, D3, D7 and D14 during the study. After 14 days, the animals were anaesthetized with ketamine (150 mg/kg) and were sacrificed. Vital organs such as the heart, lungs, kidneys, spleen and liver were removed, dissected and weighed. The relative weight of each organ was calculated.

Anti-inflammatory activities

Carrageenan-induced paw oedema

Injection of carrageenan under the plantar fascia of the hind paw of mice provokes an inflammatory response that can be reduced by any substance with anti-inflammatory properties [39].

Mice were fasted for 16 hours before testing. 0.05 mL of carrageenan (1% suspended in 0.9% NaCl) was injected under the plantar fascia of the hind paw to induce oedema in the metatarsal region. Five (05) batches of six mice were formed, including three (03) batches for the extract to be tested one (1) batch for the control and one (1) batch for the reference. The different batches were treated with the plant extract or the reference substance by gavage one hour before carrageenan injection. The

reference substance used was acetylsalicylic acid (ASA), a non-steroidal anti-inflammatory drug (NSAID) at a dose of 100 mg/kg. The negative control batch received distilled water at a 10 mL/kg dose. The herbal drug was ITM powder. The doses used for the aqueous extract of ITM were 30, 65 and 130 mg/kg bw. The initial paw volume of each mouse was measured, and oedema was induced with carrageenan 1 h after administration of the extract. Paw volume was measured at 1, 3 and 5 h after carrageenan injection. The change in volume of the treated paw makes it possible to assess the anti-inflammatory effect of each substance. The mean volume of oedema in the treated paw was calculated from 6 measurements with a maximum deviation of 4%. Anti-inflammatory activity is assessed as the percentage reduction in oedema in treated mice compared with the white control.

Lipoxygenase inhibition assay

It is an in vitro assay that determines the ability of a substance to inhibit the activity of lipoxygenase in the production of hydroperoxides. Lipoxygenase inhibition was determined spectrophotometrically by measuring the change in absorbance at 234 nm due to the formation of hydroperoxylinoleic acid from linoleic acid [40]. In a 96-well microplate, the reaction mixture contained an enzyme-blank sodium borate buffer (153.75 µL) and the LOX mixture (146.25 µL 820.51 U/mL). A mixture of 3.75 µL borate buffer, 146.25 µL LOX solution at 820.51 U/mL and 150 µL linoleic acid solution at 1.25 mM was used for enzyme activity. The extract blank consists of 146.25 µL of 820.51 U/mL L.O.X. solution, 3.75 µL of the extract and 150 µL of borate buffer. The activity of the extract consists of 146.25 µL LOX solution with a concentration of 820.51 U/mL, 3.75 µL extract and 150 µL linoleic acid solution (substrate) with a concentration of 1.25 mM. Each reaction mixture is run in triplicate, with the extract in the microplate wells. The percentage of lipoxygenase inhibition was determined by comparison with the negative control. Cascade dilutions of ITM and zileuton (reference) were performed from a stock concentration of 100 µg/mL.

Antioxidant activities

DPPH free radical scavenging assay

The DPPH antioxidant assay is based on the ability of a compound to reduce the DPPH radical. The method of Kim, 2006 was used to determine the ability of the extracts to reduce DPPH free radicals [41]. For this purpose, a concentration series was carried out using the hydro-methanolic extract of ITM at a concentration of 10 mg/mL, and Trolox 20 µL of these solutions (extract and Trolox) were added to the wells of a 96-well microtitre plate previously containing 200 µL DPPH solution (0.04 mg/mL). After incubation for 30 min, the absorbance of the mixtures was obtained at 515 nm using a microplate reader. The blank was prepared with 200 µL of DPPH and 20 µL of 99,9 % pure methanol. A curve showing the percentage inhibition of DPPH was then plotted as a function of sample concentration. This determined the concentration required to degrade 50% of DPPH (IC_{50}), which was calculated from the equation below.

ABTS radical reduction test

This test is based on the oxidation-reduction mechanism of ABTS (ammonium salt of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid). The method used was that described by Re et al. [42]. A mass of 19.2 mg ABTS plus 3.312 mg potassium persulphate was dissolved in 5 mL of distilled water. The mixture was kept at room temperature in the dark for 12 to 16 hours. A volume of 4.5 mL of the mixture was then diluted in 220 mL of analytical ethanol. Various concentration series were then prepared from a stock concentration of ITS hydroethanol extract (5 mg/mL). Trolox was used as the reference substance in this study. A 96-well microplate was used. The wells were filled with 200 µL ABTS solution mixed with 20 µL of the extract at different concentrations or with 20 µL trolox. The mixture was then incubated for 30 min at 25°C, and the absorbances were read using an Agilent 8453 UV-vis spectrophotometer at 415 nm. The white control consisted of a mixture of 20 µL 96% ethanol and 200 µL ABTS. All measurements were made in triplicate. The inhibition curve of absorbance versus extract or trolox concentration is plotted to determine the 50% inhibitory concentration (IC_{50}).

Ferric-reducing antioxidant power (FRAP) assay

The FRAP method is used to determine the chelating capacity of metals, including iron. It is based on reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The spectrophotometric method described by Hinneburg, 2006 [43] was used to assess the reducing power of ITM. To a test tube containing 0.5 mL of ITM solution (1 mg/mL), 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$, 1 %] were added. The mixture was heated to 50 °C for 30 minutes in a water bath. A 1.25 mL trichloroacetic acid solution (10 %) was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. Three aliquots of 0.625 mL were prepared in three test tubes, adding 0.625 mL of distilled water, followed by 0.125 mL of freshly prepared $FeCl_3$ (1 %) in water. A blank without extract was prepared under the same conditions. The reading was done at 700 nm against an ascorbic acid standard curve (R^2 = 0.99996). The reducing power of the extracts was expressed as ascorbic acid equivalents (AAE)/g dry extract.

Lipid peroxidation inhibition (LPO) assay

It involves inducing *in vitro* lipid peroxidation of rat liver homogenate with ferric chloride ($FeCl_2$) and hydrogen peroxide (H_2O_2). The inhibitory activity of rat liver lipid peroxidation was determined using 2-thiobarbituric acid (TBA). $FeCl_2$-H_2O_2 was used to induce peroxidation of liver homogenate according to the method of Ohkawa et al. [44]. A 0.2 mL amount of the formulation at a concentration of 1.5 mg/mL was mixed with 1 mL of 1% Wistar rat liver homogenate, then 50 µL $FeCl_2$ (0.5 mM) and 50 µL H_2O_2 (0.5 mM) were added. The mixture was incubated at 37 °C for 60 min, then 1 ml trichloroacetic acid (TCA 15%) and 1 mL 2-thiobarbituric acid (TBA 0.67%) were added and the mixture was heated in boiling water for 15 min. The absorbance was read at 532 nm using a BioRad 680 spectrophotometer. Trolox was

used as a reference. The ability of the extracts to inhibit liver lipid peroxidation was expressed as percentage inhibition.

Statistical analysis

Statistical analysis of the results of the *in vivo* tests was performed based on statistical processing using Graph Pad Prism version 10.0.2 (232) software. The One-way ANOVA statistical test, followed by Dunnett's and Student's t-test, was used to compare the extract with the references. Differences are considered significant if P (p-value) is less than 0.05 compared to the control or reference.

RESULTS

Phytochemical screening

Residual moisture content

The ITM powder's residual moisture content (RMC) was 6.36 % ± 0.05.

Phytochemical screening

Chemical characterization tests in ITM tubes revealed saponins, salt alkaloids, flavonoids, polyphenols (tannins), coumarins, sterols and triterpenes. The results are given in the table below.

Table 1 Chemical composition of the extracts of ITM

Extract	Secondary metabolites	Results
ITM	Polyphenols (tannins)	+
	Saponins	+
	Alkaloid salts	+
	Coumarins	+
	Sterols and triterpenes	+
	Flavonoids	+

(+) present; (-) absent

Thin Layer Chromatography

TLC confirmed the presence of the compounds highlighted by the colorimetric tests. The characterized metabolites are flavonoids (Figure 1a), tannins (Figure 1b), sterols and triterpenes (Fig. an c), saponins (Fig. 1 : d and e) and coumarins Fig. 1 an f), summarised in fig. 1. The chromatographic profiles are shown in Figure 1 as an example.

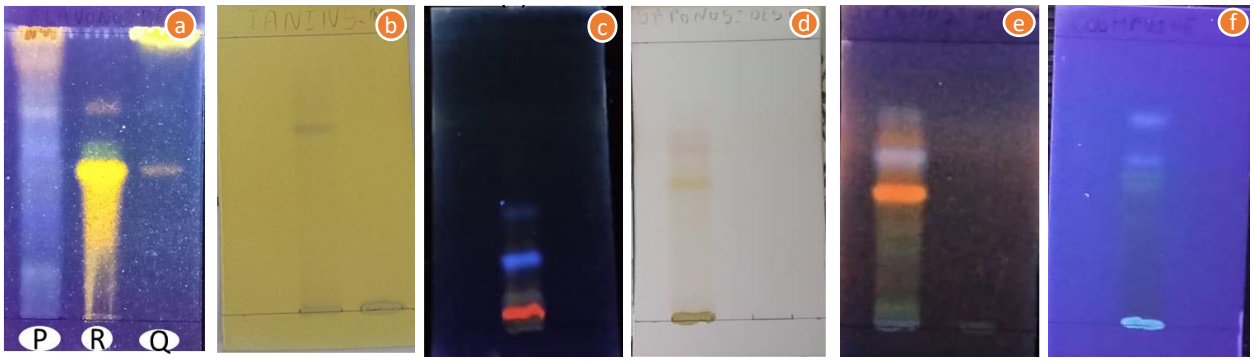

Figure 1: Analytical TLC profile revealing the presence of secondary metabolites

Legend: P = ITM; R = Rutin; Q = Quercetin

(a) Flavonoid ; (b) : Tannins ; (c) : Sterols et triterpenes ; (d) et (e) : Saponins ; (f) : Coumarins.

Reading in the visible for (b) and (d) and UV 366 nm for (a), (c), (e), et (f).

Phytochemical components contents

Total phenolics and flavonoid contents

Table 2 shows the results of the total phenolics and flavonoids assays. These results are expressed in µg tannic acid equivalent per ml extract (µg TEA/mL extract) for total phenolics and in µg quercetin equivalent per mL extract (µg QE/mL extract) for flavonoids. The total phenolic content of ITM was 50.502 ± 1.936 µg TAE/g extract and that of flavonoids was 8.017 ± 0.240 µg QE/g extract).

Table 2: Total phenolics and flavonoids contents in methanol extract of ITM

	Total phenolics (µg TAE/g extract)	**Total flavonoids (µg QE/g extract)**
ITM	50,502 ± 1,936	8,017 ± 0,240

Acute Toxicity Assay

The results of the acute toxicity study showed that administration of a single dose of 2000 mg/kg bw of ITM to female mice did not result in any notable behavioural changes or overt signs of toxicity (changes in coat, hair, eyes and mucous membranes, diarrhea, convulsion, respiratory and nervous system disturbances) during the 14-day observation period. In addition, no mortality was observed in treated rats compared with controls in the first and second tests throughout the study period.

Effects of ITM on mean relative organ weights

Fresh macroscopic observation of vital organs such as the heart, lungs, liver, kidneys and spleen of control and treated mice showed no change in the colour or appearance of these different organs. Statistical analysis by t-test showed no statistically significant difference between the mean relative organ weights of treated mice compared with controls (P > 0.05) (Table 3).

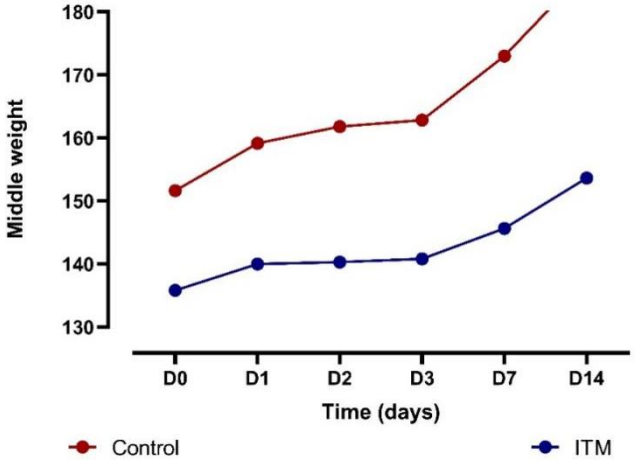

Figure 2: Evolution of the weight of the female rats in the control group in comparison with those in the experimental groups.

Table 3: Changes in the relative weights of female rat organs in the toxicity test

Organs (g)	Heart	lungs	Liver	Kidney	Spleen
Control group	0.52 ± 0.03	0.80 ± 0,04	0.84 ± 0,49	1.17 ± 0.03	0.70 ± 0.34
ITM	0.50 ± 0.04^{ns}	0.71 ± 0.05^{ns}	0.78 ± 0.41^{ns}	1.18 ± 0.07^{ns}	0.42 ± 0.05^{ns}

Anti-inflammatory activities

Carrageenan-induced paw oedema

Compared to the control, a progressive decrease in oedema volume was observed with ITM from 65 mg/kg to 130 mg/kg over 5 hours (Fig. 3).

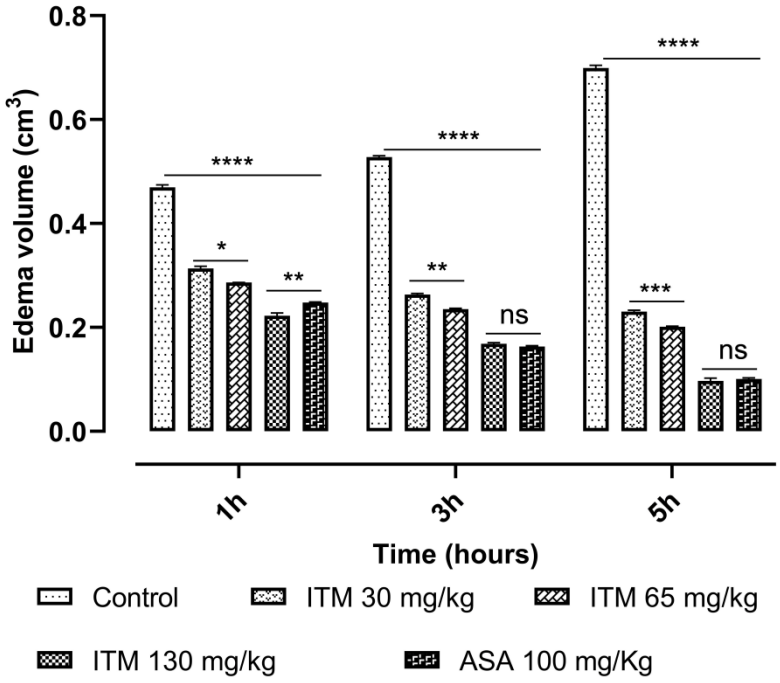

Figure 3 Effect of ITM and ASA on mouse paw oedema induced by carrageenan injection

n = 6 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ is considered significant compared to the control (Two-way ANOVA followed by "Dunnett" Multiple Comparison Test. $p < 0.05$ significant from standard. ns $p > 0.05$ is considered not significant

At T 1h after carrageenan injection, ITM at 30 mg/kg and 65 mg/kg was reduced by 32.43% and 39.37% for the two doses, respectively. At the same time, ITM at 130 mg/kg reduced the induced edema by 51.47% compared to 47.83% for the reference compound. At T 3h, all doses of ITM showed oedema inhibition percentages greater than 50%, i.e. 50.20%, 55.33% and 67.36% at 30 mg/kg, 65 mg/kg and 130 mg/kg respectively.

4.1. 15-Lipoxygenase Inhibitory Activity LOX

The percentage inhibition of lipoxygenase, a pro-inflammatory enzyme, by ITM at 100 µg/mL was 15.69 ± 0.50 % with an IC_{50} > 100 µg/mL, while that of the reference, zileuton, was greater than 50% with an IC_{50} of 2.92 µg/mL.

5. Antioxidant activity by DPPH, ABTS, FRAP and LPO methods

The antioxidant effect of the ITM was determined using the with DPPH, ABTS, FRAP and Lipid peroxidation inhibition methods (Table III)

Table 4: antioxydant effects of ITM

	DPPH	ABTS	FRAP	LPO
Products	IC_{50} (µg/mL)	IC_{50} (µg/mL)	AEAC (µM EAA/L)	% inhibition
ITM	$>10^3$	351,00 ± 7,41***	353,20 ± 1,02	81,13 ± 0,36**
Trolox	6,34 ± 0,04	3,78 ± 0,21	-	48,11 ± 3,88

IC_{50} = 50 % inhibition concentration ; AEAC= Ascorbic acid Equivalent Antioxidant Capacity ; ** = significant (p < 0,005) ; ***= highly significant (p < 0,001) of the t test.

The study of anti-radical activity by the DPPH method gave no IC_{50} values even at the ITM concentration of 10^3 µg/mL. The ABTS radical reduction method gave an IC_{50} of 351 ± 7.41 µg/mL at a concentration of 5 mg/mL for ITM and 3.78 ± 0.21 µg/mL at a concentration of 1 mg/mL for the reference, trolox. The difference was statistically highly significant. The chelating capacity of ITM from ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) expressed as µM ascorbic acid equivalent/L (µM EAA/L) was 353.20 ± 1.02 µM EAA/L. The percentage of inhibition by LPO of ITM was 81.13 ± 0.36% better than that of Trolox, which was 48.11 ± 3.88%. Trolox had the best antioxidant activity for DPPH and ABTS, but for the LPO method the activity of ITM was more pronounced.

DISCUSSION

Regarding quality control, the residual moisture content was less than 10% and could reflect good preservation of the plant drug without risk of significant changes (microbiological and chemical) [45] . Screening carried out on the ITM revealed the presence of several phytochemical groups, including flavonoids, tannins, coumarins, alkaloids, sterols and triterpenes, saponins, etc. Phytochemical analysis of the extract of *Cassia sieberiana* (a component of ITM) by Awomukwu et al. revealed the presence of alkaloids, tannins, saponins and phenols with percentages for each organ [46]. Alkaloids, anthraquinones, cardiac glycosides, flavonoids, tannins, saponins and carbohydrates were also found in *Cochlospermum tinctorium* by Musa et al. [47]. The cumulative effect of the different chemical compounds contained in the two plant powders could reduce inflammation caused by infection with various pathogens. Compounds such as phenolic compounds (tannins, flavonoids, coumarins, ...), alkaloids, triterpenes and sterols and characterized saponins are endowed with anti-inflammatory properties and molecules have been isolated from these compounds for the treatment of pathologies with inflammatory components [48–50].

The acute toxicity study test the harmful effects of an agent on the organism after a single or short-term exposure [51]. Assessment of acute systemic toxicity in rats given a lethal dose of 2000 mg per kg body weight of each extract resulted in no deaths or signs of toxicity after 14 days. According to the acute toxicity class method of OECD Test Guideline 423 and the Globally Harmonized System, the ITM extract can be classified in the 5 th toxicity class [37,52]. Therefore, the LD_{50} value was estimated to be 5000 mg/kg body weight and the extract has a relatively low acute oral toxicity.

ITM administered orally at doses of 30 mg/kg, then 65 mg/kg and finally 130 mg/kg effectively reduced carrageenan-induced inflammatory oedema in a dose-dependent manner. In the early phase, all ITM doses reduced edema, with the 130 mg/kg dose achieving 51.47% inhibition, higher than the 47.83% achieved with the reference compound, acetylsalicylic acid (ASA). The traditional practitioner recommended dose of 65 mg/kg reduced edema by 39.37%. The percentage of ITM inhibition at all doses was greater than 50% at 3 hours after edema induction. Compared with ASA, the percentage inhibition of ITM at 130 mg/kg was not statistically different from that of ASA at T 3h and T 5h after edema induction. Carrageenan-induced oedema in the mouse paw involves several mediators that induce the inflammatory response in two distinct phases [49]. An early phase, lasting approximately 1h30 after carrageenan injection, is attributed to the effect of mediators such as histamine and serotonin on vascular permeability. A late phase resulting from cyclooxygenase (COX)-mediated production bradykinin and overproduction of prostaglandins in the tissues can last more than 5 hours after carrageenan injection [53,54]. ITM inhibits the effect of mediators such as histamine,

serotonin and bradykinin on vascular permeability by acting on edema in the first few hours after induction. TMI therefore acts as a steroidal anti-inflammatory. [53,54]. Potent inhibition of oedema was observed at hour 3 for the all dose of ITM. This suggests that the inhibitory effect of ITM is exerted more on the action of prostaglandins or cyclooxygenases (COX-1 and COX-2) responsible for the biosynthesis of prostaglandins. Carrageenan-induced oedema is sensitive to cyclooxygenase (COX) and lipoxygenase inhibitors [55]. Previous work has shown that extract of *C. sieberiana* (one of the components of ITM) has anti-inflammatory activity by significantly reducing carrageenan-induced oedema in the rat paw oedema model [56]. Previous studies on *C. tinctorium* have shown that its extract significantly reduces edema induced by carrageenan, giving it anti-inflammatory properties [57,58]. The two plants act synergistically to enhance the anti-oedematous effect of ITM.

Phytochemical analysis of ITM revealed the presence of flavonoids, tannins, coumarins, sterols, triterpenes and saponins. Flavonoids and saponins are known for inhibiting pain perception and their anti-inflammatory properties [59–61]. These compounds inhibit the enzymes that produce chemical mediators of inflammation, such as arachidonic acid, prostaglandins, and pro-inflammatory enzymes (COX-1, COX-2, and LOX) [62]. Flavonoids are also thought to regulate inflammation's cellular activities (mast cells, macrophages, lymphocytes, neutrophils). Flavonoids may help to suppress or prevent the inflammatory process. As for triterpenes, they can inhibit the activity of natural kappaB (NF-κB), which plays a vital role in the inflammatory process [62,63]. These compounds could therefore inhibit inflammation in inflammatory diseases such as hepatitis. There are synergistic and antagonistic effects between the different bioactive compounds present in both plants [64,65].

The percentage of LOX inhibition was 15.69 % with an IC_{50} > 100 µg/mL compared to the reference zileuton, which was greater than 50% with an IC_{50} of 2.92 µg/mL at the single concentration of 100 µg/mL. This indicates that ITM is less active against lipoxygenase than zileuton. This ITM showed low LOX inhibitory activity, which could be explained by the fact that a mixture of raw plant powders would not use the lipoxygenase pathway. The ITM would likely act on a different enzyme, have an affinity for a different enzyme, or use a different pathway to reduce induced edema. Anti-inflammatories act either by inhibiting the enzymes (PLA_2, COX and LOX) involved in the production of pro-inflammatory mediators or by preventing the migration and activation of leukocytes at the site of inflammation. According to Macedo et al. [59], the hydroethanol extract obtained from *C. sieberiana* had an inhibitory effect on 5-LOX at concentrations ranging from 16 to 250 µg/mL and a selective inhibitory effect on COX-2 compared with COX-1. Detached quercetin appeared to partly explain the observed effects, mainly because of its significant inhibitory effects on the activity of the arachidonic acid metabolizing enzymes COX-2 and 5-LOX [59].

The antioxidant test on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical of ITM showed an IC_{50} greater than 10 mg/mL compared to the constituent plants, which had respective IC_{50} of 0.16 mg/mL for *C. tinctorium*. Perr ex Rich by Klotoé et al [56] and 22.20 ± 0.35 µg/mL for *C. sieberiana* DC by Zongo et al [66,67]. The ITM has a low capacity for scavenging free radicals H+ produced during oxidative stress. The polyphenols that inhibit the DPPH- radical were present to varying degrees in ITM. This may explain the low anti-free radical activity of ITM.

ITM also has a low antiradical capacity concerning ABTS cation radicals compared to the reference (trolox) and each plant taken individually. Indeed, the IC_{50} values of 21.43 µg/mL and 1.91 µg/mL for the rhizome of *C. tinctorium* and *C. sieberiana* leaves were reported [67]. The IC_{50} for ITM was 351 µg/mL, and for Trolox 3.78 µg/mL. This difference is probably due to the lower sensitivity of the DPPH radical reduction method, which only considers polar compounds. In contrast, the ABTS reduction method considers both polar and non-polar compounds. The chelation capacity of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), expressed in µM ascorbic acid equivalent/L (µM EAA/L), was 353.20 µM EAA/L. ITM can chelate metal ions, including ferric ions (Fe^{3+}). The reducing power of ITM is probably due to the presence of hydroxyl groups in the phenolic compounds, which can act as electron donors [68].

For LPO, ITM showed a better percentage (81.13%) than the reference Trolox (48.11%). Bioactive compounds such as polyphenols, known for their antioxidant properties, could explain these results. According to Edeas, polyphenols have antioxidant properties that essentially depend on the number and position of their functional groups and can trap the free radicals constantly produced by the body [69,70]. The presence of flavonoids could also explain the antioxidant activity of the extracts, as flavonoids are known to scavenge free radicals [71]. ITM may, therefore, have a hepatoprotective effect by helping to suppress or reduce the production of free radicals in viral hepatitis B.

CONCLUSION

Inflammation has been implicated in developing or aggravating some non-infectious and infectious diseases. Several chronic diseases such as cancer, diabetes, cardiovascular disease, autoimmune disease, neurodegenerative disease and viral hepatitis develop due to tissue lesions and genomic changes induced by persistent low-grade inflammation in and around the affected tissue or organ.

The aim of this study was to evaluate the anti-inflammatory and antioxidant properties of ITM used in the treatment of viral hepatitis B. Phytochemical screening revealed the presence of different groups of phytochemicals, including flavonoids, tannins, coumarins, saponins, alkaloids, sterols and triterpenes. The powdered ITM capsules provided good antioxidant and anti-inflammatory activity due to the presence of polyphenols and flavonoids. Phenolic compounds and flavonoids are a natural source of antioxidants and potentially active molecules in the treatment of inflammatory pathologies and/or those associated with

oxidative stress. Toxicologically, ITM has a relatively low acute oral toxicity. Chemical analysis can identify the molecule responsible for our activity and a clinical evaluation will verify the efficacy of ITM in the treatment of hepatitis B.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors' contributions

MBB carries out concept and design, experimental studies, data analysis, and writing of the manuscript, TKT and GALB contributed to the concept and to perform experimental, SRB and LT data collection and performance of the *in vitro* experiments, SO, JCRPO and SI supervised the experiments and perform *in vivo* tests, MO, DK, FBK, AT and NO supervised the work and edited the manuscript.

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