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EFFECT OF *TREMA GUINEENSIS* AQUEOUS AND ETHANOLIC EXTRACTS ON IRON, COPPER, ZINC AND MANGANESE DURING THE ACUTE INFLAMMATION AND OXIDATIVE STRESS IN RAT.

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ABSTRACT

The aim of this study was to investigate *in vivo* effects of *Trema guineensis* extracts (TGE) leaves on traces element (Fe, Cu, Mn, Zn) concentrations in carrageenan induced in rats. C-reactive protein (CRP), antioxidant marker (FRAP), Copper, zinc, manganese, iron were analyzed in blood. Firstly, we evaluated the content of trace elements (Cu, Zn, Fe, Mn) in the *Trema guineensis* leave extracts. The ethanolic extract was richer in Cu and in Fe. Then TGE (200 mg/kg), drugs references (oligosol Cu/Zn: 0.50 mg/mL and oligosol Mn: 0.59 mg/mL) were administered to rats (N = 6) by intraperitoneal (0.5 mL). One hour after drug treatment all animals were injected with 0.2 mL of 1% suspension of carrageenan in normal saline into the subplanter region of left hind paw to induce inflammation and oxidative stress. Trace elements (Cu, Zn, Mn, Fe) were measured by flame atomic absorption spectrophotometer 5 hours later and compared to healthy controls (NaCl 0.9%). Mn, Cu, Fe and Zn concentrations varied significantly among the different groups. The ethanolic extract administering and the standard drug favored obtaining better trace elements contents. The CRP and oxidative stress by FRAP test in animals received carrageenan had confirmed its role in the installation of both pathologies *in vivo*. This analysis showed enough the plant extracts possess the anti-inflammatory and antioxidant activities. The analysis of iron showed that both extracts are rich in nonheminic iron.

KEYWORDS: *trema guineensis*, oligosol, trace elements, carrageenan, Côte d'Ivoire.

INTRODUCTION

The use of plant extracts for medicinal purposes has been going on for thousands of years; and it has been the source of much useful therapy in both herbalism and folk medicine.[1] According to World Health Organization (WHO) 80% of the world population uses plants extracts to cure different diseases because of their effectiveness with no harmful side effects, low cost, easy access, ancestral experience and high cost of other forms of treatments.[2,3] Yet many of them possess untapped potential by the scientific community.

That is the case of *Trema guineensis* which is widely used in folk medicine for the treatment of diseases, such as malaria and Hypertension.[4] The role of elements in health and disease is now an established fact.[5, 6] Trace elements are virtually crucial to all biochemical and physiological process in plants, animals and human beings.[7] Among these, iron, zinc, cobalt, manganese, nickel, copper, chromium and molybdenum are now thought to be essential for human's life.[8] Plants are also the source of these minerals.[9,10] Trace elements are usually structural compounds, cofactors for protein and especially more enzymes involved in the anti-radical defenses. Some such as zinc, iron, manganese, copper are needed to maintain the human health as they have the ability to counteract free radicals and protect the macromolecules against oxidative damage.[11,12] It has been also demonstrated those mentioned trace elements have an effect on inflammation.[13] That's why the present study was designed to investigate the *in vivo* effect of these plant extracts on the trace elements concentrations in carrageenan induced in rats, after determining TGE composition itself in trace elements.

MATERIAL AND METHODS

Drugs and reagents

Carrageenan (HiMedia Lab. Pvt. Ltd. Mumbai, Indian), Ferric Reducing Ability of Plasma (FRAP), Sodium acetate, 2,4,6-tripyridyl-striazine (TPTZ), glacial acetic acid, $FeCl_3$*$6H_2O$; HCl (Merck Co. Germany).

Guanidine Hydrochlorure, Ascorbate, FerroZine (trademark of Hach Chemical Co., Ames, lowa), NaCl, Oligosol (Labcatal, industrial area of White Mount-France), Ethanol (100%), Hemafer® (Uni-Pharma Kleon Tsetis Pharmaceutical laboratories S.A). Plug TRIS with bovine serum albumin and immunoglobulines, Particles of latex covered with anti-CRP antibody, sodium azide with 0.09% provided by Rock Diagnoses too. All other chemicals and reagents used were of analytical grader and obtained from standard sources.

Plant material

The fresh leaves of *Trema guineensis* were collected in Abobo (Abidjan) in 2012. The plant species was later identified and authenticated by the department of Botany, FHB University of Abidjan. They were further dried at room temperature under the shade for two weeks and pulverized using the crushing assistance (IKAMAG RCT®). The powder of leaves obtained, constituted our sample to be analyzed.

Instrumentation

Trace elements were analyzed by flame atomic absorption spectrophotometer (FAAS) with a SpectrAA 20 (Varian Techtron, Springvale and Australia).

CRP concentration was measured with Cobas C-311, a Roche modular analyzer (Roche Diagnostics Corp., Indianapolis, IN, USA) as indicated in the manufacturer's instructions.

The absorption measurements were performed under the conditions recommended by the manufacturer. The crushing assistance (IKAMAG RCT®) was used for the pulverization of TGE. Spectrophotometer and furnace muffle were allowed successively to measure antioxidant parameter (FRAP) and seedling powders mineralization (Naberthem-Germany).

Aqueous extract preparation

Trema guineensis powder was used to prepare the various extracts. 100g of the powder was extracted in 1L of distilled water. The mixture obtained was then homogenized using a mixor during 24 hours. The homogenate obtained is filtered successively twice on absorbent cotton then once on Wattman N°1 filter paper. The filtrate was carried thereafter to evaporation in a drying oven with 50°C during 48 hours. We obtained this way a powder which constituted the aqueous total extract used for the preparation of various concentrations of products.[14]

Ethanolic extract preparation

100g of *Trema guineensis* powder were extracted in one liter (1L) of ethanol-water mixture (70/30, v / v). Following unfolds as aqueous extraction.[15]

Aqueous and hydroalcoolic extracts obtained starting from these leaves powders were used to carry out our studies.

Experimental animals

Adults Wistar Albinos rats of either sex, weighing 150-300g were procured from animal house. These animals were kept in animalery of the Training and Research Unity of Pharmaceutical and Biological Sciences at FELIX Houphouet Boigny University. The rats were fed with FACI (Fabrication d'Aliments de Côte d'Ivoire) pellets, groundnuts and dried fish. Their drink was tap water. A total of 48 rats were used in this study. The rats were housed in standard cages at constant temperature of 22±1°C and relative humidity 55±5% with 12h light-dark cycle for, at least 1 week before the experiments.

The care and the conditions of animals' treatment are in conformity with the hot lines of the Organization for Economic Cooperation and Development.[16]

In vitro determination of minerals content

The solid sample (*Trema guineensis* residue obtained after extraction) has undergoes initially mineralization by calcination. On Sartorius analytic balance (England), were weighed 0,3g of *Trema guineensis* powder in a porcelain crucible of 30 mL; This test specimen was calcined in furnace muffle (Naberthem-Germany) regulated with 600°C during 5 hours; after cooling, 5 mL of 1N nitric acid was added then evaporated dry on sand bath (or hot plate). To the residue, was added 5mL 1N hydrochloric acid and the whole was charged again with 400°C during 30 min. The residue was recovered in 10mL of 0,1N hydrochloric acid and then was put into 50 mL volumetric flask. The operation was repeated three times and the flask was supplemented to the feature of gauge. The elements contained in the solution were then assayed by AAS and the results were expressed as follows.[17]:

$$T = \frac{Cess - Cbl}{Pess} X\, V$$

C_{ess}: Sample concentration (mg/mL)
C_{bl}: Element concentration in the extraction solution (control) in mg/mL
P_{ess}: Test specimen (Kg)
V: Test recovery volume (mL)
T: content (µg/g or mg/Kg)

Carrageenan induced in rats

The animals were equally divided into six groups (n = 6) each kept in separate cages. On the day of the experiment, the rats assigned as vehicle and healthy (Group 1) received only saline water. The negative control group (Group 2) received NaCl at first.

The groups 3 and 4 received 200 mg/kg of *Trema guineensis* aqueous and ethanolic extracts respectively. In the group 5, the animals were treated with reference drug (oligosol® Zn/Cu: 0.50 mg/mL) in the study of *in vivo* inflammation and oxidative stress biomarker (CRP and FRAP). We use the same group (group 5) as a positive control drug for the proportioning of Zn and Cu. Group 6 constituted the positive control group for Mn proportioning. Animals of group 7 were treated with another positive control drug (hemafer®: 50mg/mL) for proportioning of iron.

All drugs were administered by intraperitoneal with the same volume (0.5 mL) and 60 min after drug treatment, 0.2 mL of 1% suspension of carrageenan in normal saline was administered into the subplanter region of left hind paw of animals. After 5 h of carrageenan administering, all animals were sacrificed and blood samples were collected into heparin-treated collection tubes. The plasma was separated to be used later.[18, 19]

Blood Analysis

The concentrations of those markers were measured at the same time as the plasma trace elements concentrations. Blood analysis included trace elements: iron, zinc, copper, iron and manganese; it concerned also C-reactive protein (CRP) and the total antioxidant capacity of plasma by FRAP method.

In order to proportion trace elements, plasma was separated and de-proteinisation was done by placing 1.0 mL of plasma in the test tube and adding 3 mL of 2 M HCl.[20] The clear supernatant was aspirated into the flame atomic absorption spectrophotometer (AAS) with a SpectrAA 20 (Varian Techtron, Springvale, and AUS) after adjusting the wavelength at 324.8 nm, 248.3 nm, 279.5 nm and 213.9 nm for copper, iron, manganese and zinc respectively.

This model flame atomic absorption spectrophotometer (FAAS) equipped with hollow cathode lamps was used for trace elements determinations. The acetylene–air flame in the FAAS was used as described in the manufacturer's instructions for the spectrophotometer.

The optimum working was 0.02–5 µg/mL for Mn 0.1–24 µg/mL for Cu, 0.01–2 µg/mL for Zn and 0.06–15 µg/mL for Fe.

The concentrations were displayed electronically and the results were expressed in mg/L.

FRAP (Ferric Reducing Ability of Plasma) test as described by Benzie and Strain (1996).[21]

The CRP was determinated by turbidimetric method, as described by Wick *et al*. (1996) and Kleeberg (1975).[22,23]

Statistical Analysis

The values expressed as Mean ± SEM from 6 or 5 animals. The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnet,s t-test, $p < 0.05$ were considered as significant.

RESULTS

As reported in the **table 1**, the *Trema guineensis* extracts (TGE) contained trace elements: Zn, Cu, Mn and Fe.

Table 1: Composition of *Trema guineensis* extracts in trace elements.

Elements	Content (µg/g of dry extract)	
	Aqueous extract	Ethanolic extract
Fe	3 ± 0.05	7 ± 0.15
Zn	2 ± 0.8	4 ± 0.21
Cu	6 ± 0.13	7 ± 0.10
Mn	2 ± 0.10	1 ± 0.09

The experiment was performed 3 times and results are expressed as mean ± standard deviation.

The **Table 2** indicated CRP and FRAP values.

Table 2: *In vivo* anti-inflammatory and oxidative stress biomarkers of TGE leaves in rats

Groups N=6 animals	Dose / Concentration ($g.kg^{-1}$b.wt or mg/mL)	CRP (mg/L)	FRAP (µmol of iron II / L)
AQ. Ext + carrageenan	200	0.25 ± 0.17**	619.7 ± 1.53***
Eth. Ext + carrageenan	200	0.18 ± 0.04***	730 ± 5.00***
NaCl	-	0.06 ± 0.02***	409 ± 2.52***
NaCl + carrageenan	-	0.44 ± 0.03	302.7 ± 4.73
Oligosol® + carrageenan	0.50	0.31 ± 0.12*	508 ± 3.81***

CRP and FRAP values are means ± SEM (standard error of the mean) with n= 6; *P < 0.05, **P<0.01, ***P<0.001 compared successively to NaCl + carrageenan.

Trace elements content in the treated and untreated rats were shown in table 3.

Table 3: Trace elements contents in plasma from rats treated with TGE (*Trema guineensis*) extracts, at the dose of 200 mg/kg).

Groups N=6 animals	Dose / Concentration ($g.kg^{-1}$b.wt or mg/mL)	Trace elements			
		Cu (mg/L)	Mn (mg/L)	Zn (mg/L)	Fe (mg/L)
NaCl	-	3.81 ± 0.10**	0.41 ± 0.08**	3.20 ± 0.15***	25.51 ± 0.26*
NaCl + carrageenan	-	1.07 ± 0.18	0.15 ± 0.3	0.99 ± 0.17	12.50 ± 0.18
Aq Ext + carrageenan	200	6.80 ± 0.53 ***	0.33 ± 0.2*	4.70 ± 0.21 ***	25.88 ± 0.52*
Eth Ext + carrageenan	200	7.41 ± 0.68 ***	0.64 ± 0.27 ***	5.58 ± 0.14 ***	32.82 ± 0.23***
Oligosol® Cu/Zn + carrageenan	0.50	8.23 ± 0.17 ***	0.94 ± 0.15 ***	7.18 ± 0.20***	-
Oligosol® Mn + carrageenan	0.59				-
Hemafer® + carrageenan	50	-			46.01± 0.36***

Results are expressed as mean ± standard deviation with n= 6; *P < 0.05, **P<0.01, ***P<0.001 compared successively to NaCl + carrageenan.

DISCUSSION

From the table 1, it can be seen that the manganese and the zinc content were the lowest in the extracts. The Fe content was the highest in aqueous extract (3 ± 0.05 µg/g) and in ethanolic extract (7 ± 0.15 µg/g) it was still the highest. Copper is much more present in both extracts. So TGE contains the studied trace elements in different concentrations.

The anti-inflammatory activity was performed by the carrageenan test and the determination of CRP concentration.[24] The rat paw edema induced by this phlogogenic agent (carrageenan) was considered as an inflammation sign.[25] Carrageenan-induced rat paw edema model is a suitable test for evaluating anti-inflammatory drugs. It is a strong chemical use for the release of inflammatory and pro-inflammatory mediators such as prostaglandins, leukotrienes, histamine, bradykinin and TNF-α.[26] The course of acute inflammation is biphasic. First phase starts with the release of histamine, serotonin and kinins after the injection of phlogistic agent in the first few hours.[27] While the second phase is related to the release of prostaglandins like substances in 2-6 hours, second phase is sensitive to both the clinically useful steroidal and non steroidal anti-inflammatory agent.[28,29] Prostaglandins are the main culprit responsible for acute inflammation.

The presence of micronutrients in the two extracts could confer antioxidant and anti-inflammatory properties to the plant.

Concerning the table 2, the ethanolic extract administering had considerably reduced the value of CRP (0.18 ± 0.04mg/L) at the 5^{th} hour compared to control group (NaCl + carrageenan). The aqueous extract and oligosol® decreased also the value of CRP (0.25 ± 0.17mg/L and 0.31 ± 0.12mg/L) at the same time but they influenced the inflammation with a less degree. TGE might be containing some anti-inflammatory agents which are responsible for the blockage of prostaglandins and inflammatory pathway.

During this inflammatory phase, the *in vivo* concentrations of trace elements such as Cu, Mn and Zn (table 3) of both extracts caused to drop considerably compared to their value in the case of the healthy rats group. Yet, the values of these elements increased significantly when the inflammation was treated with the aqueous and ethanolic extracts compared to the same control group (NaCl+ Carrageenan). The standard drug (oligosol®) used, fostered also a significant increase in trace elements.

According to Douart (1994) and Loriol (2001) three modes of actions explain the importance of trace elements *in vivo*.[30,31] They have an enzymatic action, hormonal action and another action on the ionic channels. Increasing the zinc concentration may be related to the contribution of ethanolic and aqueous extracts. This mineral would have an influence on the inflammation because according to Lucie Burdin, 2014 zinc allows the synthesis of prostaglandins and leucotriens by eactivating the phospholipase A_2, lipo-oxygenase and the cyclo-oxygenase.[32]

Copper has anti-infectious and anti-inflammatory role starting from its particular chemical properties. In our study, this essential trace element would have contributed to the fall of inflammation because during this process, copper inhibits the production of pro-inflammatory cytokines.[33] Manganese would inhibit the calcium channel and block the potassium output; its action is exerted on bronchial muscle fibers and the cells secreting histamine. That indicates its importance in the inflammation.

The present data demonstrated that TG extract had a powerful iron chelator; it induced a significant reduction of inflammation and lipid peroxides due to its antioxidant and free radical scavenger properties.

The results obtained in oxidative stress (table 2) showed that the ethanolic extract (730 ± 5.00µmol of iron II/L) of TG exhibited higher antioxidant potential than aqueous extract (619.7 ± 1.53 µmol of iron II/L).

The reducing properties are associated with the presence of compounds which exert their action by the free radical chain breaking through donating a hydrogen atom.[34]

The studied of trace elements would play an important role in this antioxidant activity. Zinc possesses significant antioxidant and acts according to several mechanisms:
- It is the cofactor of superoxide dismutase, key enzyme trapping the superoxide ions.
- It has a direct anti-radicalizing action on the hydroxyl radical formation. It can be also opposed to the non-enzymatic reactions catalyzed by iron (Reaction de Fenton) producing the hydroxyl radical.[35]

As for copper, it is also co-factor of dismutase superoxide (Cu/Zn-SOD 1 and 3), key enzyme in protection against the free radicals. Copper provides the catalytic functions of enzyme SOD by stimulating it in order to thwart free radicals production.[36]

In biology, iron and manganese play important roles in oxygen chemistry. Both trace elements serve as cofactors for enzymes that remove harmful products of O_2 metabolism such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide. Manganese can safely operate as cofactor for superoxide dismutase (SOD).[37]

CONCLUSION

The use of medicinal plants plays a vital role in converting the basic health needs and these plants may offer new source of antioxidant compounds which possess anti inflammatory.

The TGE pretreatment of rats appears to prevent the carrageenan induced alterations of some trace elements such as Cu, Zn, Mn directly or indirectly implicated in the antioxidant and anti-inflammatory systems. The ethanolic extract provides more trace elements and would protect the cells from damage oxidative stress and inflammation. This study enabled us to know that the *Trema guineensis* ethanolic extract had a high potential of storage and transfer of iron. This plant would be useful for people suffering from inflammatory anemia. The presence of the polyphenols in both extracts would have strongly contributed to trace elements influence in anti-inflammatory and antioxidant activities.

Abbreviation

Aq: Aqueous
Ext: Extract
Eth: Ethanolic
b.wt: body weight

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