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Anti-Inflammatory and Antioxidant Activities of Ethanol and Aqueous Leaves Extract of *Trema guineensis*

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ABSTRACT

Trema guineensis is a plant of Cote d'Ivoire which is widely used in folk medicine for the treatment of a variety of diseases such as malaria, anemia. We have tested the anti-inflammatory activity (inhibition of carrageenan induced paw edema, erythrocyte sedimentation test, concentration of Reactive protein-C), the *in vitro* antioxidant (DPPH radical essay and lipid peroxidation) and the *in vivo* antioxidant activities (FRAP, TBARS essay) of its ethanolic leaves extract as well as its aqueous leaves extract. The maximum inhibition (33.58%) was obtained with the ethanolic extract with the dose of 200 mg kg^{-1} b.wt. after 5 h of drug treatment in carrageenan induced paw edema, whereas diclofenac (used as the standard) produced 27.97% of inhibition. Reactive Protein-C (CRP) concentration indicated that the ethanolic extract (0.29±0.02 mg L^{-1}) had more impact on the edema. This tendency was still observed with the erythrocyte sedimentation test. Linear regression analysis was used to calculate IC_{50} value. The results showed that, the ethanolic extract exhibited significant DPPH with IC_{50} value of 20.23±0.4 µmol mL^{-1} while, the aqueous extract inhibited with IC_{50} value of 12.3±0.09 µmol mL^{-1} in comparison to the control (vitamin C). Lipid peroxidation induced by the Fe^{2+}, was inhibited more by the ethanolic extract. The *in vivo* study in rats with the extracts showed a significant antioxidant activity using the FRAP and TBARS methods. The ethanolic extract values (11.77±0.33% and 730±5.00 µmol of iron II/L) were the best compared to the vitamin C (reference molecule). These observations helped us to conclude that the ethanolic and aqueous extracts are endowed with interesting anti-inflammatory and antioxidant activities; in addition, the ethanolic extract (200 kg mg^{-1} b.wt.) was most active. The phenols, the flavonoids and the flavonols may play an important role in these activities.

Key words: *Trema guineensis,* inflammation, digital caliper, antioxidant, carrageenan

INTRODUCTION

Inflammation is a primary defense mechanism that helps physiological body to protect itself from infection, toxic chemicals or noxious stimuli. Recent studies indicate that the picks and cellular effectors of inflammation are major constituents of the local environment of tumors as indicated by Mantovani *et al*. (2008).

Inflammation and pain are a common feature in almost all non-communicable diseases (Prescott, 2013). These disorders can also be traumatic, especially for people whose occupations expose them to these inflammatory and pain syndromes. This inflammation is also characterized by oxidative damage (Young *et al*., 2008; Sander *et al*., 2004). The reactive oxygen species play an important role related to the degenerative or pathological processes of the inflammation (Aruoma, 1998). Current treatment for this disease involves the steroidal anti-inflammatory drugs (glucocorticoids) and drugs, such as aspirin. While, effective these molecules usually have side effects that can hinder their long-term use as indicated by Phillipson and Wright (1991). Yet, indigenous plants play an important role in the treatment of many diseases and 80% of people world wide are estimated to use herbal remedies (Graziano *et al*., 1976).

Moreover, in developing countries, plants with anti-inflammatory and antioxidant activities could provide an alternative to the anti-inflammatory therapy because of their greater accessibility and their lower toxicity in general, compared to conventional anti-inflammatory drugs as recommended by Yen and Duh (1994).

In Cote d'Ivoire, many herbal extracts and natural products prevent or reduce oxidative stress and inflammatory (Bagul *et al*., 2005). It is the case of *Trema guineensis* which is widely used in folk medicine for the treatment of a variety of diseases, such as malaria and anemia. The antioxidant and anti inflammatory properties of this plant have not yet been pharmacologically evaluated; so that is unknown by the scientific community. Hence, the present study was undertaken to evaluate antioxidant and anti-inflammatory activities of ethanolic and aqueous leaves of *Trema guineensis*.

MATERIALS AND METHODS

Materials

Plant material: The fresh leaves of *Trema guineensis* were collected in Abobo (Abidjan) in 2012. The plant material was identified by Professor Ake Assi, a plant taxonomist in the Laboratory of Vegetable Biology (Felix Houphouet Boigny University). They were dried away from the sun during two weeks and pulverized using the crushing assistance (IKAMAG RCT®).The powder of leaves obtained, constituted our sample to be analyzed.

Drugs and reagents: Carrageenan (HiMedia Lab. Pvt. Ltd. Mumbai, Indian), Diphenyl-picryl-hydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), gallic acid, Folin- Ciocalteu reagent and methanol (Merck Co. Germany), Quercetin (Sigma Chemical Co.; St. Louis, USA). Sodium acetate,2,4,6-tripyridyl-striazine (TPTZ), 2-thiobarbituricacid (TBA), 1,1,3,3-tetramethoxypropan (MDA), Trichloro Acetic Acid (TCA), glacial acetic acid, $FeCl_3$,$6H_2O$; HCl and n-butyl alcohol (Merck Co. Germany). All other chemicals and reagents used were of analytical grader and obtained from standard sources.

Aqueous extract preparation: The powder of *Trema guineensis* was used to prepare the various extracts. Hundred gram of the powder was extracted in 1 L of distilled water. The mixture obtained was then homogenized using a mixor during 24 h. The homogenate obtained is filtered successively twice on absorbent cotton then once on Wattman No. 1 filter paper. The filtrate was carried thereafter to evaporation in a drying oven with 50°C during 48 h. We obtained this way a powder which constituted the aqueous total extract used for the preparation of the various concentrations of the products (Guede-Guina *et al*., 1993).

Ethanolic extract preparation: Hundred gram of *Trema guineensis* is powder were extracted in 1 L of ethanol-water mixture (70/30, v/v). Following unfolds as aqueous extraction (Zirihi *et al*., 2005). Aqueous and ethanolic extracts obtained starting from these powders of leaves were used to make the studies of anti-inflammatory and antioxidant activities.

Experimental animals: Adults Wistar Albinos rats of either sex, weighing 100-300 g 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 Cote d'Ivoire) pellets, groundnuts and dried fish. Their drink was tap water. A total of 116 rats (Edema: 36, CRP: 30, speed of sedimentation: 30 and 20 animals for the *in vivo* antioxidant test) were used in this study. The rats were housed in standard cages at a constant temperature of 22±1°C and relative humidity 55±5% with 12 h 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 (OECD., 2001).

Blood collection: For this study, the total volume of blood which can be taken with an animal (example of the rat or the mouse) is not enough. Thus, the number of taking away was distributed on several animals. We did not take with an animal more than 20% of its total blood volume.

The volume of taken blood took into account the weight and the total volume of blood available to the animal. About 3 mL of blood was collected from puncturing the retro orbital sinus from anesthetized rats. The blood was centrifuged at 2000×g for 10 min to separate serum. This serum was kept at -20°C until the analysis (OVF., 1981).

Anti-inflammatory activity
Experimental animals:

- **Carrageenan induced paw edema model:** Anti-inflammatory activity was assessed by the method described by Winter *et al*. (1962) and Chithran *et al*. (2012). The albinos rats of either sex weighing were divided in 6 groups (N = 6). Group 1 received the saline solution (control), group 2 received diclofenac which is the reference standard with 25 mg kg^{-1} b.wt. as recommended by Bashir and Qureshi (2010). Group 3 and 4 received ethanolic extracts 100 and 200 mg kg^{-1} b.wt. of *Trema guineensis*, respectively. Group 5 and 6 received the second extract (aqueous) with amounts 100 and 200 mg kg^{-1} b.wt. All the animals were treated with drugs by intraperitoneal route and subsequently 1 h after treatment; 0.2 mL of 1% suspension of carrageenan in normal saline was administered into the subplanter region of left hind paw to induce edema (Majid *et al*., 2012; Ravi *et al*., 2009). The paw diameter was measured initially at 0, 1, 2, 3, 4, 6, 24 and 48 h after carrageenan injection using a Digital Caliper (digital paw edema meter). The difference between the initial and subsequent values provided the actual edema diameter which was compared with positive control
- **Collection of blood for erythrocyte sedimentation:** The blood was collected by the method of gauged and graduated tube, as described by Westergren (1957)
- **Collection of serum for reactive protein-C test (Concentration of CRP):** The rats were divided into 5 groups (N = 6). The vehicle group (Group 1) received normal saline 0.9% (v/v).

The negative control group (Group 2) received NaCl+Carrageenan. The positive control group (Groups 3) was treated with the standard drug, diclofenac (25 mg kg^{-1}). The tested groups (4 and 5) received 200 mg kg^{-1} b.wt. of ethanolic and aqueous extracts. All the doses were administered 60 min before the induction of edema by administering 0.2 mL of carrageenan. After 5 h of carrageenan administering, all animals were sacrificed and blood samples were collected; the serum was separated to be used later (Suralkar *et al*., 2012)

- **Essay procedure of rat reactive protein-C in serum:** The CRP in serum was determinated by the turbidimetric method, as described by Wick *et al*. (1996) and Kleeberg (1975)

Antioxidant activity

Polyphenolic components

Determination of total phenolic contents: Total phenols were determined by Folin-Ciocalteu method, as indicated by McDonald *et al*. (2001).

Estimation of total flavonoids content: The total flavonoids content was determined according to the method of Rao *et al*. (2012).

Estimation of total flavonols content: Total flavonols were determinated by the method of Miliauskas *et al*. (2004).

***In vitro* antioxidant activity**

Inhibition lipid peroxidation determination: Lipid peroxidation was determinated by Fenton method, as described by Choi *et al*. (2002).

DDPH radical assay: The free radical scavenging activity of the extracts and vitamin C were measured with the DPPH method as indicated by Parejo *et al*. (2000). The percentage inhibition of DPPH radical by the samples was calculated according to formula of Yen and Duh (1994).

The IC_{50} value was determined from the plotted graph of scavenging activity against the different concentrations of *Trema guineensis* extracts, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. The measurements were triplicated and their scavenging effect was calculated based on the percentage of DPPH scavenged.

***In vivo* antioxidant activity**

Experimental design

Carrageenan induced hind paw edema to generate the stress: The stress was generated by induction of the carrageenan to the leg postpones right rats. The albinos rats of either sex weighing were divided in 4 groups (N = 6). Group 1 received the saline solution (control), group 2 received vitamin C (reference standard), group 3 and 4 received ethanolic and aqueous extract (200 mg kg^{-1} b.wt.) of *Trema guineensis* and NaCl+Carrageenan, respectively. All the animals received 1 h after treatment, 0.2 mL of 1% suspension of carrageenan into the subplanter region of left hind paw to induce edema. The blood collected 6 h after, is centrifuged and the serum is used for the antioxidant activity study.

Lipid peroxidation assay: Lipid peroxidation was determinated by Thiobarbituric Acid Reactive Substances (TBARS) method, as described by Satoh (1978).

Total antioxidant power assay: The total antioxidant capacity of serum was determined by measuring its ability to reduce Fe^{3+}-Fe^{2+} by the FRAP (Ferric Reducing Ability of Plasma) test as described by Benzie and Strain (1996).

Statistical analysis: The values expressed as Mean±SEM from 6 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

Plant yield: The yield of the *Trema guineensis* leaves ethanolic and aqueous extracts were 10.55 and 14.98%, respectively.

Anti-inflammatory activity

Erythrocyte sedimentation test: The erythrocytes sedimentation of the group NaCl+Carrageenan was fastest. The aqueous extract presented a strong significant difference while the extract ethanolic presented a weak significant difference in comparison to the diclofenac. These results are indicated by Fig. 1.

Carrageenan-induced paw edema: The illustration of values reduction in paw diameter is in Table 1. The ethanolic extract of the plant caused significant reduction in inflammation 33.58% (200 mg kg^{-1}) followed by aqueous extract 7.59% (200 mg kg^{-1}) compared to standard anti-inflammatory drug, diclofenac sodium 27.97% (25 mg kg^{-1}). The Table 2 indicated the percentage inhibition of extracts.

Concentration of Reactive Protein-C (CRP): The CRP concentration, 5 h after carrageenan induction enabled us to obtain the results indicated by Fig. 2. The concentrations of CRP obtained were not significant with the ethanolic extract and the vehicle (NaCl only) except for the aqueous extract and NaCl+Carrageenan.

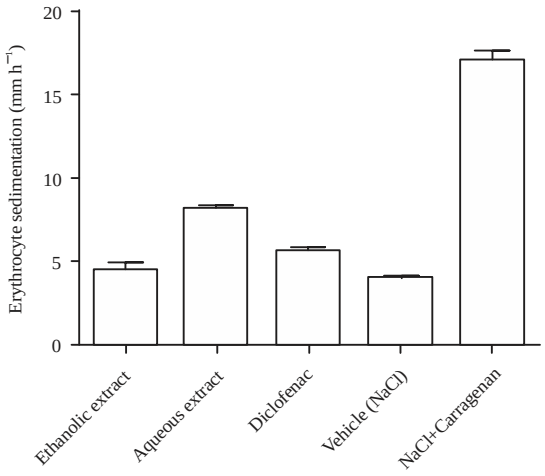

Fig. 1: Erythrocyte sedimentation of the ethanolic and aqueous extracts compared to negative and positive control

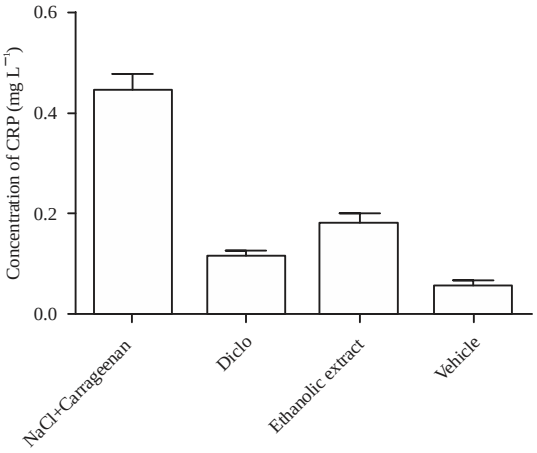

Fig. 2: Effect of *Trema guineensis* on CRP concentration 5 h after carageenan injection

Table 1: Effect of *Trema guineensis* leaf extracts on carrageenan induced paw edema in rats

	Edema diameter (mm)								
Treatment groups (n = 6)	0 h	1 h	2 h	3 h	4 h	5 h	6 h	24 h	48 h
NaCl	4.05±0.11	7.02±0.22	7.65±0.07	7.94±0.16	8.10±0.08	7.90±0.10	7.82±0.27	6.35±0.11	4.32±0.09
Diclo	4.15±0.09	6.05±0.32^{c}	6.17±0.39^{c}	6.24±0.39^{c}	5.84±0.38^{c}	5.69±0.39^{c}	5.60±0.42^{c}	5.00±0.42^{c}	4.20±0.11
AQ 100	4.12±0.06	6.63±0.13^{b}	7.35±0.15	7.79±0.06	7.69±0.05^{b}	7.53±0.13^{a}	7.45±0.12^{a}	6.03±0.08^{c}	4.19±0.02
AQ 200	3.98±0.45	6.33±0.04^{c}	7.18±0.21^{a}	7.59±0.07^{a}	7.50±0.19^{c}	7.40±0.20^{c}	7.30±0.04^{b}	6.02±0.05^{c}	4.11±0.33
ETH 100	4.01±0.08	5.80±0.12^{c}	6.00±0.38^{c}	6.10±0.07^{c}	5.68±0.05^{c}	5.55±0.11^{c}	5.41±0.17^{c}	4.23±0.04^{c}	4.14±0.06
ETH 200	3.99±0.07	5.55±0.14^{c}	5.84±0.04^{c}	5.89±0.09^{c}	5.38±0.05^{c}	5.33±0.05^{c}	5.28±0.07^{c}	5.01±0.08^{c}	4.05±0.05

AQ 100: Aqueous extract (100 g kg^{-1} b.wt.), AQ 200: Aqueous extract (200 g kg^{-1} b.wt.), ETH 100: Ethanolic extract (100 g kg^{-1} b.wt.) ETH 200: Ethanolic extract (200 g kg^{-1} b.wt.), Diclo: Diclofenac (25 m g kg^{-1} b.wt.), Each value is Mean±SEM N = 6 rats, ^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001, One way ANOVA followed by Dunnet multiple comparison test, statistically significant when compared to control (NaCl)

Table 2: Percentage inhibition of aqueous and ethanolic leaves extracts of *Trema guineensis* on rat paw edema induced by carrageenan

	Inhibition (%) (h)								
Treatment	1 h	2 h	3 h	4 h	5 h	6 h	24 h	48 h	Mean of inhibition (%)
Normal saline (Control)	-	-	-	-	-	-	-	-	-
AQ 100	5.56	3.92	2.63	5.06	4.68	4.73	5.51	4.19	4.39±1.00
AQ 200	9.83	6.14	5.13	7.41	6.33	6.65	5.51	5.09	6.51±1.56
ETH 100	3.42	3.92	6.88	7.41	21.27	21.87	4.09	3.00	8.98±2.93
ETH 200	20.94	23.66	26.38	33.58	32.53	32.48	21.26	6.25	24.63±3.98
Diclofenac	13.82	19.34	22.00	27.90	27.97	28.39	21.26	2.78	20.43±3.75

Antioxidant activity

Quantitative analysis of antioxidant components: The total phenolic contents in the aqueous and ethanolic extracts of *Trema guineensis* are shown in Fig. 3. The aqueous extract possessed high phenol contents (8.84±0.21 mg g^{-1} gallic acid equivalent) followed by the ethanolic extract with flavonoids (4.26±0.06 mg g^{-1} quercetin equivalent) and flavonols (1.05±0.015 mg g^{-1} quercetin equivalent). Phenolic compounds, especially flavonoids and phenols have been shown to possess significant values in ethanolic extract.

In vitro antioxidant activity

DPPH test: The values of IC_{50} of the extracts tested, calculated with the linear line of regression compared to the standard were: ethanolic extract (IC_{50}) was 12.0±0.09 μg mL^{-1}, aqueous extract (IC_{50}) was 20.23±0.48 μg mL^{-1}. The curves indicating the amount-answers of the ethanolic extract and the vitamin C are presented in Fig. 4.

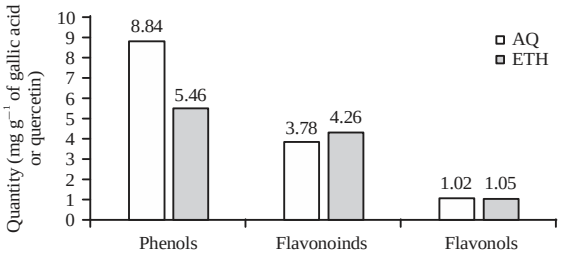

Fig. 3: Polyphenolic contents of *Trema guineensis* ethanolic and aqueous extract

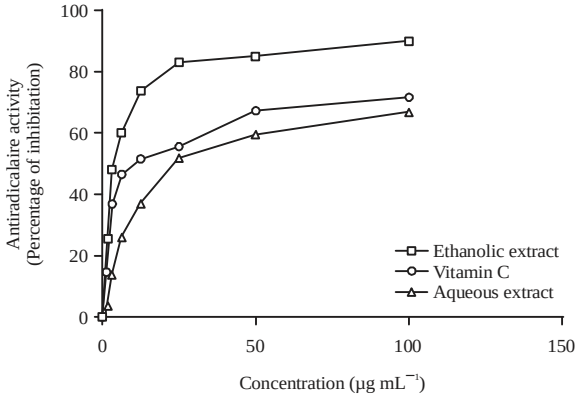

Fig. 4: Antiradicalaire activity of *Trema guineensis* (ethanolic and aqueous extracts)

Table 3: *In vivo* antioxidant activity of *Trema guineensis* leaves extracts by FRAP and TBARS methods

Treatments	FRAP (µmol of iron II/L)	TBARS (%)
Aqueous extract	619.7±1.53***	23.94±0.06***
Ethanolic extract	730.0±5.00***	11.77±0.33***
Vitamin C	619.0±1.00	49.63±0.34
NaCl	409.0±2.52***	65.03±0.06***
NaCl+Carrageenan	302.7±4.73***	33.13±1.02***

Values are Means±SEM (standard error of the mean) with n = 6, *p<0.05, **p<0.01, ***p<0.0001compared to vitamin C

Lipids peroxidation: The dose 200 mg kg^{-1} b.wt., enabled us respectively to obtain the lipid peroxidation of the hydro-alcoholic extract, the aqueous extract and the vitamin C as follow: 65.92±0.20, 36.38±0.442 and 54.83±0.0360 µmol L^{-1}.

In vivo antioxidant activity

Total antioxidant power assay by FRAP and TBARS assay: The FRAP values are presented in the Table 3; the values of the ethanolic extract and the vitamin C are much higher compared to control NaCl.

The results of the reducing activity of the tested extracts of *Trema guineensis*, the vitamin C, the vehicle and NaCl+Carrageenan were also indicated in Table 3. The antioxidant activities of both extracts are statistically more marked in comparison to the vitamin C. However the ethanolic extract possessed the greatest value in the two methods.

DISCUSSION

Inflammatory activity: Carrageenan-induced rat paw edema is a prominent inflammatory model to evaluate anti-inflammatory effect of compounds (Di Rosa *et al*., 1971). Inflammatory reactions are generally triggered off by exogenous or endogenous aggressions which are characterized by vascular and cellular events.

Carrageenan induces an inflammatory reaction in different phases. The initial phase, which occurs between 0 and 2 h after injection of carrageenan, has been attributed to the release of histamine, serotonin, bradykinin, protease, prostaglandin and lysosome on vascular permeability (Vinegar *et al*., 1987). Inflammation diameter reaches its maximum approximately 3-4 h post treatment after which it begins to decline (Garcia *et al*., 2004). That was confirmed with our two extracts. The late phase, which is also a complement-dependent reaction, has been shown to be due to overproduction of prostaglandin in tissues (Vinegar *et al*., 1969). Among the ethanolic (100 and 200 mg kg^{-1} b.wt.) and aqueous (100 and 200 mg kg^{-1} b.wt.) extracts, the plant extract with dose 200 mg kg^{-1} caused maximum inhibition of paw edema by 32.53%, 4 h after carrageenan administering (Table 2). This value was significant compared to the diclofenac (27.9%).

Therefore, it can be assumed that the inhibitory effect of the extract of plant on carrageenan-induced inflammation could be due to the inhibition of the enzyme cyclooxygenase, leading to the inhibition of prostaglandin synthesis (Das *et al*., 2009). The values of the CRP concentration and the VS have showed that the ethanolic extract and the molecule of reference, strongly support inhibition while the action of the aqueous extract is weak. These results were confirmed the anti-inflammatory activity of *Trema guineensis* extracts.

Antioxidants activities

***In vitro* antioxidant activity:** Lipid peroxidation cause oxidative damage not only in food system but also in human body (Esaki *et al*., 1997). Peroxyl radical is a key step in lipid peroxidation and is an important cause of tissue damage and thus, oxidative stress (Halliwell, 1995). Antioxidant may offer resistance against oxidative stress by inhibiting lipid peroxidation and some other mechanisms (Youdim and Joseph, 2001). The activity of the aqueous extract was better than the ethanolic extract activity.

The DPPH is stable nitrogen centered free radical which can be effectively scavenged by antioxidants and shows strong absorbance at 517 nm. The change in absorbance of DPPH radical caused by the extracts was due to the reaction between the antioxidant molecules and the extracts, which resulted in the scavenging of the radical by hydrogen donation. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of IC_{50} values (Villano *et al*., 2007). Our ethanolic and aqueous (200 mg kg^{-1} b.wt.) extracts showed significant DPPH scavenging activity (IC_{50} 20.23±0.48 and 12.00±0.09 μg mL^{-1}), respectively when compared with the IC_{50} values of the standard ascorbic acid (IC_{50} = 5.03±0.056 μg mL^{-1}). The ethanolic extract has higher DPPH scavenging activity in comparison to aqueous extract.

***In vivo* antioxidant activity:** The antioxidant activity *in vivo* of *Trema guineensis* after inflammation induction in rats was assessed from serum TBARS and the concentration iron ions III reduced iron ions II.

Sharma and Gupta (2008) noted that the antioxidant activity can be attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction and the reductive capacity.

Earlier authors have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts (Tanaka *et al*., 1998). The presence of reducing agents is thought to be associated with the reducing properties (Duh *et al*., 1999) which in turn have been shown to exert an antioxidant action by donating a hydrogen atom that breaks the free radical chain (Gordon, 1990).

In our case, the results obtained indicated a good reductive activity of both extracts higher than obtained with the vitamin C (positive control).

CONCLUSION

The results of the study have shown that the ethanolic leaves extract of *Trema guineensis* with amount 200 mg kg^{-1} b.wt., possess significant anti-inflammatory effects and antioxidant activity compared to aqueous extract. We can then say that the ethanolic extract would possess the best activity. It may be assumed that these properties could be due to the presence of various flavonoids and related polyphenols. These preliminary results lend to support to the use of this plant in folk medicine for inflammation. Therefore, further investigation is needed to clarify the exact active components responsible for anti-inflammatory action and their mechanism of action as well as the enzymes implied in the production of reactive oxygenated species.

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