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# Subacute Toxicity, Subacute Anti-inflammatory and Anti-arthritic Activities of Combination of Hydroethanolic Extract of *Terminalia macroptera* and *Ximenia americana In-vivo*

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**The aim of this study was to assess the anti-inflammatory effects of a combination of medicinal plants on two models of inflammation. Subacute toxicity was assessed by daily oral administration of 2000 mg/kg body weight (bw). Subacute inflammation and arthritis were induced using the carrageenan air pouch granuloma model and Complete Freund's Adjuvant (CFA) respectively. After 28 days of administration, the combination at 2000 mg/kg proved to be non-toxic and induced a significant reduction ($p<0.05$) in transaminases and total cholesterol. The combinations C3 (150 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana*), C2 ((250 mg/kg of *T. macroptera* + 150 mg/kg of *X. americana*) and C1 (250 mg/kg of *T. macropteria* + 250 mg/kg of *X. americana*) inhibited fresh granuloma formation by 40.37, 45.63 and 58.32% and dry granulomas by 47.77, 55.08 and 61.24% respectively. The combinations significantly ($p<0.001$) reduced air pouch fluid volume and massive leukocytes infiltration compared with the control group. With regard to the anti-arthritic effect, the combination C1 showed significant inhibition ($p<0.05$) of primary and secondary lesions compared with the control CFA. The increase in serum ALT, AST and uric acid concentrations observed in the CFA control group was significantly reduced ($p<0.001$) by the combination C1. An antioxidant effect was observed with the administration of the combination C1 and prednisone, which resulted in a significant increase ($P<0.01$) in GSH, SOD and catalase activity and a decrease in MDA concentration ($P<0.001$) compared with the CFA control group.The results suggest that the combination C1 has anti-inflammatory and anti-arthritic effects and prevents oxidative stress in arthritic rats.**

**Keywords**: *Terminalia macroptera*; *Ximenia americana*; Combination, anti-inflammatory effect, anti-arthritic effects, oxidative stress.

Inflammation is intended to repair tissue damage. The immune system fights this damage by inducing acute inflammation within minutes or longer [1]. When the tissue damage has not recovered, acute inflammation turns into chronic inflammation [2]. Inflammation is initiated by a local increase in vessel diameter and high permeability, resulting in inflammatory oedema following

passage of exudate. Subsequently, leukocytes migrate into the damaged tissue and eventually a granuloma is formed [3]. These inflammatory leukocytes have degranulation and superoxide production as responses. These products generate free radicals or reactive oxygen species, which cause oxidation and attack cell membranes and biomolecules, making them dangerous. Oxidative stress is the consequence of the imbalance between antioxidants and oxidants, causing alterations in proteins and lipids [3,4]. This situation potentiates inflammation, especially in chronic pathologies, which are a public health problem [5].

Drugs to treat inflammatory have serious adverse effects or are very expensive. This reflects the interest of researchers in therapeutic substances derived from plants as a source of molecules to prevent or treat inflammatory diseases [6].

In West Africa, medicinal plants, accessible, less toxic and effective inflammation could be a possibility alongside conventional medicines [7,8]. The *Terminalia macroptera* leaves and *Ximenia americana* roots are indicated in several inflammatory diseases according to traditional medicine. Ethnobotanical studies have revealed that in Burkina Faso, Mali and Guinea, the two plants mentioned above are prescribed against hepatitis, tuberculosis, wounds and inflammations [9–11]. Both plants have also shown anti-inflammatory activities *in vitro* and anti-inflammatory effects against acute inflammation models. Both plants also showed *in vitro* anti-inflammatory activities and anti-inflammatory effects against acute inflammation models [12,13]. The aim of this study was to assess the subacute anti-inflammatory and anti-arthritis effects of the combination.

## MATERIALS AND METHODS

### Plant samples

*Terminalia macroptera* leaves and *Ximenia americana* roots were harvested in September 2020 on the hill of an outlying (Samé) district of Bamako. The plants were identified at the Department of Traditional Medicine and herbarium of each plant was deposited under number 2468; 0027 respectively. The collected samples were dried in the drying room of the Department of Traditional Medicine at room temperature and protected from sunlight. They were then pulverized.

### Extraction methods

On each powder, two extraction methods at 10% (m/v) were performed. Aqueous decoction and hydroethanolic (30:70 v/v) maceration were used as described previously [14]. briefly, 100 g of powder in 1 liter of 70% ethanol macerated for 24 hours and for decoction, 100 g of powder in 1 liter of distilled water, boiled for 15 minutes.

### Experimental animals

Male and female wistar rats of 8-10 weeks, from the Department of Traditional Medicine were used. Rats were placed in cages at a temperature of 24 ± 2 ºC. At 12/12 h of light and dark. All experiments were conducted in accordance with international animal care guidelines [15]. All described procedures were reviewed by the Ethics Committee and a protocol approval was issued #Reg. No. 2021 / 234 / USTTB.

### Reagents and Medication

The following products were used: Carrageenan SIGMA-ALDRICH; Adjuvant Freund's

Complete SIGMA-ALDRICH, Ellman's reagent; trichloroacetic acid; thiobarbituric acid; dichromate; acetic acid; adrenaline; sodium diclofenac and prednisone.

### Subacute toxicity

Subacute toxicity was performed according to OECD Test Guideline 407 [16]. 30 rats including 15 males and 15 non-pregnant nulliparous females aged 8 weeks were used. Rats were randomized into 3 homogeneous groups of 5 males and 5 females each, treated daily by

### Gavage for 28 consecutive days

Group 1: neutral control, 10 µl/g of distilled water was administered;

Group 2 and group 3 (satellite): 2000 mg/kg bw of the combination was administered;

After 28 days of treatment, the satellite group rats were observed for two weeks without any administration. To monitor for possible reversibility, persistence or late onset of toxic effects. On day 29 (two weeks later for the satellite group), the rats were sacrificed and blood was collected from dry and EDTA tubes. Biochemical and hematological parameters were measured.

### Carrageenan granuloma inflammation model (air pouch)

The method previously used by DA et *al.*[17] was slightly modified for this study. Twenty-

five rats randomized into 5 groups of 5 for the experiment. They were anaesthetized by injection of ketamine (100 mg/kg) intraperitoneally on day 0 and the pouch was created by subcutaneous administration of 10 ml of sterilized air to dorsal surface. On third day, each air pouch was re-inflated with 6 ml of sterile air and immediately, 4 ml of 2% carrageenan in 0.9% NaCl introduced. The animals received daily oral treatment for 4 days.

Group 1: negative control, 10 µl/g of distilled water was administered;

Group 2: positive control, 50 mg/kg bw diclofenac was administered;

Group 3: combination C1: 250 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana* was administered;

Group 4: combination C2: 250 mg/kg of *T. macroptera* + 150 mg/kg of *X. americana* was administered;

Group 5: combination C3: 150 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana* was administered.

On 7th day, the rats were sacrificed. The exudate was aspirated, measured and used to quantify leukocytes. Granuloma, liver and blood in dry EDTA tubes were collected.

**Arthritis induced in rats by Complete Freund's adjuvant (CFA)**

The anti-inflammatory activity of the combination against chronic inflammation in rats was evaluated according to the method previously described [18]. The rats were randomized into 4 different groups of 5 rats (n = 5):

Group 1 (neutral control): no CFA injection and rats received distilled water;

Group 2 (CFA control): CFA injection and rats received distilled water;

Group 3 (positive control): CFA injection and rats received 5 mg/kg/day of prednisone;

Group 4: CFA injection and rats received 500 mg/kg/day of combination C1.

On first day, the volumes of the hind legs were recorded and 100 µl of CFA was administered subcutaneously to the left hind paw. The animals were treated for the first twelve days. The effect against the primary lesion of the prednisone and the combination was assessed by measuring the volume of the paw injected on the 5th day. On the 21st day, the volume of the posterior paws was measured; afterwards the rats were sacrificed, liver and blood were collected to assess biochemical and oxidative stress parameters [19,20]. The arthritic index was assessed macroscopically. The morphological features of polyarthritis were scored from 0 to 4 [21].

For primary lesions: The percentage inhibition of the injected paw volume versus control was measured on 5th day. Secondary lesions were estimated by the increase in volume of the non-injected paw versus control on the last day of the experiment. The total of the scores corresponds to the arthritis index.

**Preparation of serum and liver homogenate**

The serum of the collected blood was kept in the refrigerator for biochemical examinations. After dissection, the liver was removed and washed immediately with cold 0.9% NaCl. 0.20 g of ground liver was homogenized in 1 ml of 50 mM Tris-HCl and centrifuged.

**Biochemical assays**

AST, ALT, total cholesterol and triglyceride levels were measured according to the procedures described in the commercially available reagent kit.

**Estimation of oxidative stress biomarkers**

The supernatant was used to determine oxidative stress parameters. Catalase (CAT) activity was determined by the colorimetric method according to Sinha [22]. Superoxide dismutase was estimated to assess the ability to inhibit auto-oxidation accord [23]. The concentration of malondialdehyde (MDA) was assessed and reduced glutathione (GSH) was estimated [24,25].

**Statistical analysis**

Results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's test for Graph Pad Prism® version 5.03. Differences were considered statistically significant, very significant and highly significant when p was <0.05 (*), <0.01 (**) and <0.001 (***), respectively.

## RESULTS

**Sub-acute toxicity**

The results focused on biochemical and hematological parameters.

**The effects of the combination on hematological constants**

Hematological parameters such as white and red blood cells, hemoglobin, platelets and

hematocrit showed no significant variation between the different groups (table 1).

**Effects of the combination on biochemical parameters**

The biochemical profiles of the different groups of rats are presented in table 2. The combination at 2000 mg/kg induced a significant ($p < 0.05$) decrease in transaminases and total cholesterol. This decrease disappeared after an additional two weeks observation without administration in the satellite group. However, no variation of kidney markers such as creatinine and urea were observed in neither the combination group or satellite group.

**Table 1.** Effect of the combination on hematological parameters of different groups of male and female rats

| Hematology parameters | Control | Combination 2000 mg/kg bw | Satellite |
|---|---|---|---|
| Males | | | |
| WBC ($10^9$/l) | 16.52 ± 1.56 | 15.24 ± 2.59 | 15.86 ± 2,06 |
| RBC ($10^{12}$/l) | 8.32 ± 1.06 | 8.97 ± 0.23 | 8.58 ± 0.31 |
| HGB (g/dl) | 15.07 ± 1.27 | 15.33 ± 0.93 | 14.95 ± 1.02 |
| PLT ($10^9$/l) | 844.2 ± 46.08 | 858.4 ± 32.39 | 847 ± 37,85 |
| HCT (%) | 44.36 ± 4.24 | 46.72 ± 2.52 | 46.34 ± 3.31 |
| Females | | | |
| WBC ($10^9$/l) | 15.84 ± 1.34 | 15,44 ± 4,14 | 16,04 ± 1,87 |
| RBC ($10^{12}$/l) | 9.08 ± 0.16 | 9,32 ± 0,53 | 9,17 ± 0,58 |
| HGB (g/dl) | 15.59 ± 0.79 | 15,78 ± 1,27 | 15,45 ± 0,36 |
| PLT ($10^9$/l) | 858.6 ± 34.77 | 869,2 ± 35,81 | 857,8 ± 27,91 |
| HCT (%) | 44.94 ± 1.23 | 46,56 ± 4,18 | 47,88 ± 3,39 |

The values are expressed as mean ± SEM, n = 10. WBC: White blood cells, RBC: Red Blood Cell, HGB: Hemoglobin, PLT: Platelets and HCT: hematocrit.

**Table 2.** Effect of the combination on the biochemical parameters of the different groups of male and female rats

| Biochemical parameters | Control | Combination 2000 mg/kg bw | Satellite |
|---|---|---|---|
| Males | | | |
| Creatinine (µmol/l) | 51.26 ± 2.75 | 54.2 ± 3.07 | 52.86 ± 3.29 |
| Urea (µmol/l) | 4.38 ± 0.63 | 4.87 ± 0.57 | 4.83 ± 0.63 |
| Cholesterols (mg/dl) | 76.2 ± 2.63 | 70.93 ± 6.21* | 75.45 ± 4.52 |
| Triglycerides (mg/dl) | 35.94 ± 3.46 | 37.64 ± 3.17 | 36.71 ± 2.8 |
| AST (U/l) | 47.09 ± 1.98 | 43.63 ± 1.64* | 45.54 ± 1.52 |
| ALT (U/l) | 42.2 ± 1.44 | 40.30 ± 3.16 | 40.72 ± 1.91 |
| Females | | | |
| Creatinine (µmol/l) | 50.48 ± 2.66 | 54.99 ± 2.89 | 51.78 ± 1.38 |
| Urea (µmol/l) | 4.67 ± 0.89 | 5.18 ± 0.53 | 4.72 ± 1.09 |
| Cholesterols (mg/dl) | 78.89 ± 3.1 | 72.25 ± 3.1* | 76.27 ± 3.03 |
| Triglycerides (mg/dl) | 36.61 ± 1.35 | 37.5 ± 2.38 | 35.83 ± 1.11 |
| AST (U/l) | 49.3 ± 2 | 46.5 ± 1.37* | 47.4 ± 1.33 |
| ALT (U/l) | 43.42 ± 1.56 | 41.54 ± 1.77 | 41.37 ± 1.05 |

The values are expressed as mean ± SEM, n = 10. * $p < 0.05$ when comparing the other groups to the control group

**Effects of the combination on carrageenan-induced inflammation in the air pouch**

**Effect of the combination on air pouch granuloma**

Figure 1 shows a significant ($p<0.001$) decrease in inflammatory granuloma formation by all three combinations (C1, C2, C3) compared to the negative control. This decrease was also significant ($p<0.001$) for dry granuloma weight. Furthermore, a significant decrease ($p<0.05$) in the dry granuloma weight of rats in the diclofenac group was observed compared to rats of combination C2 group.

**Effects of the combination on exudate volume and leukocyte infiltration in the air pouch**

Inflammation produced a significant ($p<0.001$) increase in exudate volume and leukocyte infiltration in the negative control group compared to rats in all three combinations (C1, C2, C3) and diclofenac. The volume of exudate and leukocyte infiltration of rats in the diclofenac group did not differ significantly from rats in the combination C1 (Figure 1).

**Effects of the combination C1 on Arthritis**

For primary lesions, prednisone 5 mg/kg and the combination C1 showed significant inhibition ($p < 0.05$) of oedema compared to the CFA control.

However, the effect on primary lesions of the combination was inferior to that of prednisone. On the last day of treatment ($12^{th}$ day), prednisone and combination C1 significantly ($p < 0.01$) inhibited the oedema of the injected paw compared to the negative group. As for secondary lesions, prednisone and combination C1 showed significant ($p < 0.05$) inhibition of non-injected paw volume compared to CFA control. The percentage inhibition of non-injected paw volume was maximal for the combination C1. Arthritis scores were significantly ($P < 0.01$) elevated in CFA control compared to combination C1 group. (Table 3).

**Effects of the combination C1 on biochemical parameters**

The combination C1 significantly reduced serum ALT, AST, creatinine and urea concentrations compared to CFA control. Furthermore, this

**Table 3.** Anti-inflammatory activity of the combination C1 on chronic inflammation by CFA-induced arthritis in rats

| Groups | Increase in paw volume (Mean ± SEM) (ml) (% Inhibition within parentheses) | | | | |
|---|---|---|---|---|---|
| | | Injected paw | | Uninjected paw | Arthritis Index |
| | Day 5 | Day 12 | Day 21 | Day 21 | Day 21 |
| CFA control | 3.85 ± 0.9 | 3.17 ± 1 | 1.97 ± 0.7 | 0.44 ± 0.2 | 7.1 ± 0.22 |
| Prednisone | 2.41 ± 0.3** (33.34%) | 1.78 ± 0.3** (40.21%) | 0.51 ± 0.3*** (74.26%) | 0.15 ± 0.1* (64.63%) | 4.2 ± 0.84*** |
| Combination C1 | 2.75 ± 0.5* (25.64%) | 1.82 ± 0.1** (38.68%) | 0.55 ± 0.3** (71.76%) | 0.13 ± 0.1* (73.47%) | 4.6 ± 1.14** |

The values are expressed as mean ± SEM, n = 5. ***p < 0.001, **p < 0.01, *p < 0.05 Significant different when compared CFA to combination C1 and prednisone.

**Table 4.** Effect of combination C1 on biochemical analysis of CFA-induced arthritis in rats

| Biochemical parameters | CFA control | Combination C1 | Prednisone |
|---|---|---|---|
| Creatinine (µmol/l) | 109.7 ± 1.5 | 104.1 ± 0.8* | 103.3 ± 1.5* |
| Urea (µmol/l) | 7.8 ± 0.6 | 5.5 ± 0.6* | 5.2 ± 0.3** |
| Uric acid (µmol/l) | 508.4 ± 2.7 | 372.9 ± 1.21***, # | 302.2 ± 1.5*** |
| AST (U/l) | 93.85 ± 2.4 | 61.4 ± 1.7*** | 67.74 ± 1.2*** |
| ALT (U/l) | 78.6 ± 6.4 | 44.6 ± 1.2*** | 47.1 ± 2.2*** |

The values are expressed as mean ± SEM, n = 5. ***p < 0.001, **p < 0.01, *p < 0.05 and #p < 0.05 Significant different when compared CFA control to treatments and prednisone to combination C1 respectively.

reduction was not significantly different from the prednisone group. Uric acid concentration was also significantly ($p < 0.001$) reduced by the combination and prednisone. Prednisone produced a significant reduction ($p < 0.05$) compared to the combination C1 (Table 4).

**Effects of the combination C1 on oxidative stress biomarkers**

The results showed a significant ($P<0.001$) elevation in MDA levels, a significant ($P<0.001$) reduction in GSH levels, SOD and catalase activity in the CFA control compared to the neutral control. The combination C1 and prednisone reversed this trend by causing a significant ($P < 0.01$) elevation in GSH, SOD and catalase levels and a reduction in MDA level ($P < 0.001$) compared to CFA control (Figure 3).

## DISCUSSION

The aim of this study was to assess the subacute anti-inflammatory and anti-arthritis effects of the combination. The anti-inflammatory effect was observed by a significant reduction ($p<0.05$) in granuloma formation, exudate volume and leucocyte infiltration in the air pouch. The administration of plant extracts would affect the physiology of many organs such as the liver and

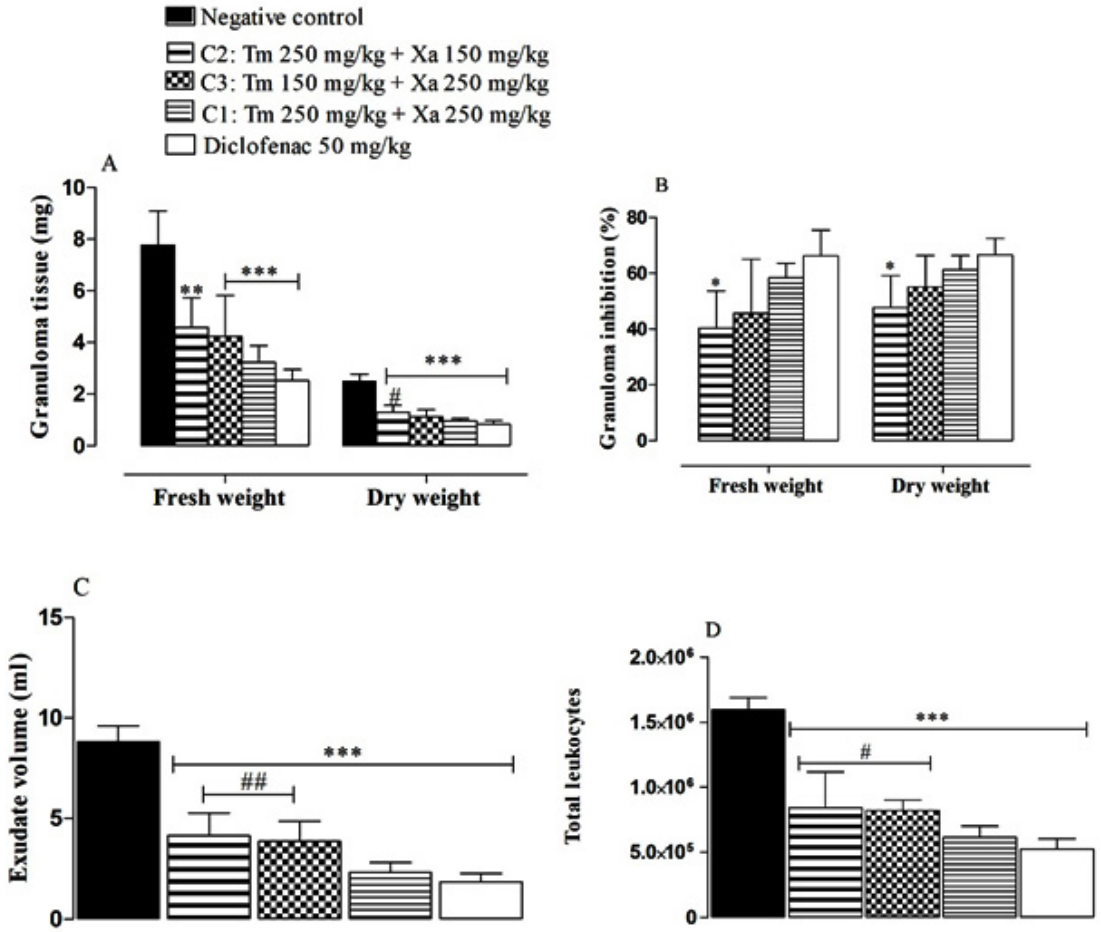


**Fig. 1.** Effects of the combination and diclofenac on capillary permeability and leukocyte recruitment of air pouch inflammation. A: Effect of the combination on the fresh and dry weight of granuloma tissue; B: Effect of the combination on granuloma tissue; C: Effect of the combination on exudate volume; D: Effect of the combination on leukocyte infiltration of the exudate. The values are expressed as mean ± SEM, n = 5. *p< 0.05, **p< 0.01, ***p< 0.001 significant difference when compared to negative control and #p< 0.05, ##p< 0.01 significant difference when compared to diclofenac.

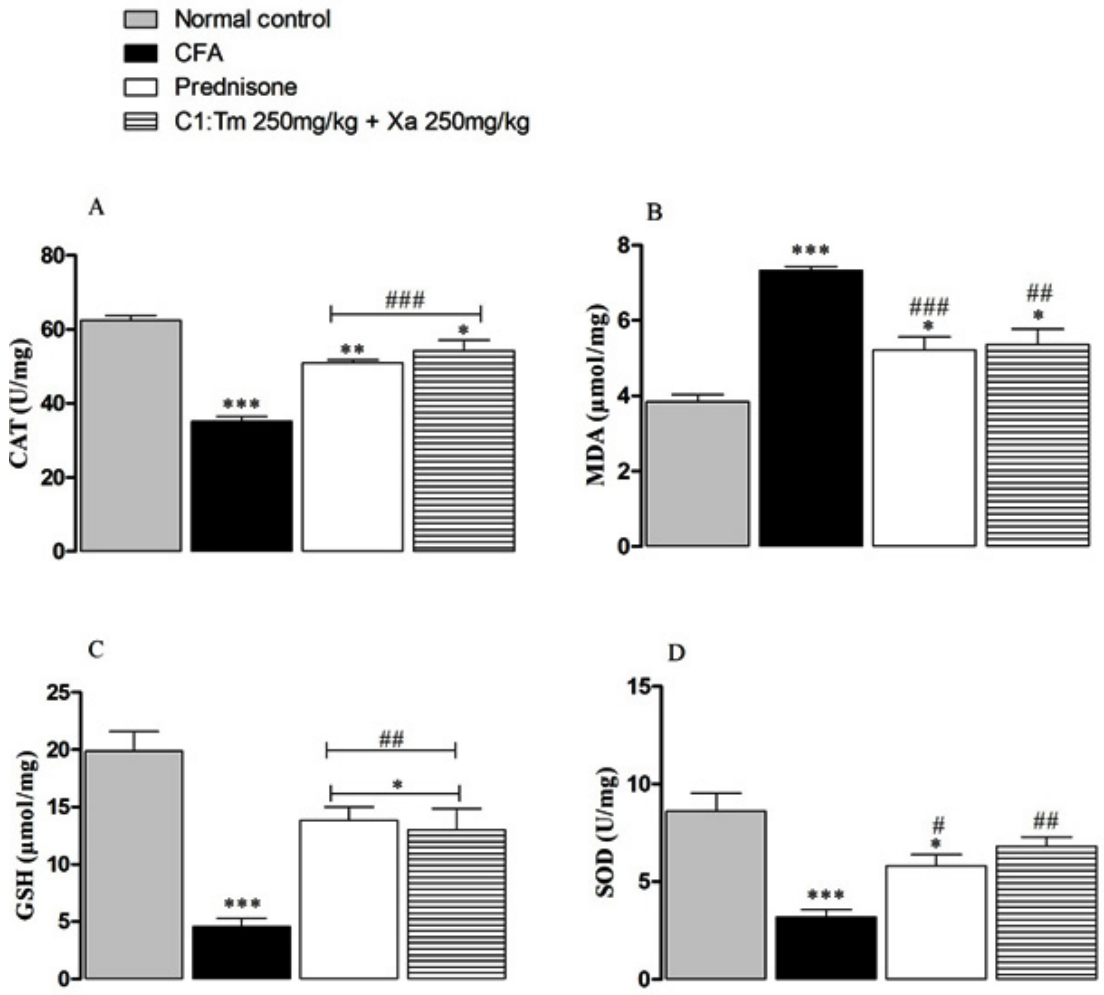


**Fig. 2.** Effects of the combination and prednisone on oxidative stress parameters. The values are expressed as mean ± SEM, n = 5. *p< 0.05, **p< 0.01, ***p< 0.001 significant difference when compared to negative control group

kidneys. It is important to carry out a biochemical assessment in a sub-acute toxicity study [26]. Biochemical assessments included blood levels of creatinine, urea, transaminases, cholesterols and triglycerides. The only changes observed were in ALT and cholesterol. The combination induced a significant decrease ($p < 0.05$) in blood levels of ALT and cholesterol. Similar results were obtained with extracts of *Celosia trigyna* and *Eleophorbia drupifera* leaves in rats [26,27]. ALT and cholesterols are considered to be a more specific indicator of the hepatoprotective effect and lipid balance respectively, which would reflect the existence of hepatoprotective properties of the combination. The main endogenous markers of renal function are creatinine and urea. Their increases or decreases may reflect, respectively, renal failure or muscle atrophy [28]. Our results showed no significant change with the administration of 2000 mg/kg of the combination in male and female rats. Other authors have found similar results [29]. Serum AST, ALT, cholesterol and triglyceride levels and urea and creatinine levels were analyzed to identify possible hepatic and renal damage due to sub-acute treatment, a critical point for the development of new analgesic or anti-inflammatory drugs [30]. The results suggest that the combination is not nephro- or hepatotoxic under our test conditions.

In inflammatory responses, an acute phase with vasodilatation and high permeability, subacute phase with cell migration and a chronic phase, where fibrosis of the tissues is noted[31]. Subacute and chronic inflammation models such as air pouch were used to assess the transudative and proliferative components of inflammation [17]. The results showed that granuloma formation was inhibited by the combinations. This inhibition followed this order: diclofenac > C1 > C3 > C2. Diclofenac showed significant inhibition ($p < 0.05$) compared to C2. The volume of fluid in the pouch was also significantly reduced by the combinations. C1 showed similar inhibition to diclofenac.

This reduction in granuloma formation and transudate would demonstrate the power of the extracts to inhibit the synthesis of macro-molecules and prevent the formation of granulomatous tissue [17,32]. The getting leukocytes to the site of inflammation is an important parameter of the inflammatory response. Leukocyte migration results from an elaborate series of events, including cell adhesion and motility [3,33]. The combinations inhibited the accumulation of leukocytes in the inflammatory fluid of the pouch. The results showed that the combinations especially combination C1 has effects on this carrageenan granuloma air pouch model and could therefore be a potential source of drugs against sub-chronic inflammation.

Polyarthritis is a chronic inflammatory illness that injures the joints and causes deformity, disability and premature death in most patients [34].

The induction of arthritis in rats, previously described [18] manifests itself as in humans. This model is frequently used to examine the effect of drugs against polyarthritis. After inoculation into rats, CFA induces polyarthritis in two phases. The acute phase, a maximum after 3 to 5 days, is attributed to the primary lesions, while the chronic phase occurs after 11 to 12 days, attributed to the secondary lesions and recognized by inflammation of the non-injected paw [21]. In primary lesions, CFA-injected rats showed a significantly ($P<0.05$) reduced paw volume with the administration of combination C1. Arthritis scores were significantly ($P < 0.01$) elevated in the CFA control compared to combination C1 group. This suggests that the combination has anti-inflammatory properties and the ability to attenuate immune system responses. In the 19th century, Garrod discovered that hyperuricemia was the cause of gout and it is clear that serum uric acid may play a key role in inflammatory responses [35,36]. The combinations significantly reduced ($P < 0.001$) the increase in serum uric acid levels.

Imbalance between oxidants and antioxidants induces hepatic damage causing lipid alterations that result in increased MDA levels. Antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase convert reactive oxygen species into harmless compounds that inhibit lipid peroxidation [37]. The induction of arthritis even alters the antioxidant defense system, nearly depleting the vital lines of defense (GSH, SOD and CAT) against reactive oxygen species [38]. Increased serum ALT and AST levels confirm liver damage. These were observed in the CFA control group. These results are in line with the results of previous studies [39–41]. The combination C1 induced a significant ($P < 0.01$) increase in catalase activity, SOD and reduced glutathione accompanied by a significant ($P < 0.01$) decrease in MDA levels compared to CFA control. Similar results were also reported by other investigators [42–44]. These results show that the combination C1 can reduce the deleterious effects of oxygen radical accumulation. Combination C1 therapy could be an alternative to combat oxidative stress. Previous studies have shown high contents of total polyphenols, flavonoids and tannins in the two hydroethanolic extracts of the two plants that form the combinations [14]. Effect of the combinations on the two models of inflammation used in this study could be explained by the presence of total polyphenols, more precisely tannins, and the capacity of these two hydroethanolic extracts that form the combinations to inhibit pro-inflammatory enzymes. The antioxidant power of the combination is attributed to the flavonoids. [12,13].

## CONCLUSION

Finally, the non-toxicity of the combination after daily administration of a dose of 2000 mg/kg for 28 days. The combination showed potential action against sub-acute inflammation and markedly reduced the symptoms of CFA-induced arthritis. The combination showed a significant decrease in inflammatory granuloma formation, exudate volume and leukocyte infiltration. The

combination also significantly reduced the concentration of uric acid. Furthermore, the combination significantly prevented oxidative stress in arthritic rats. We can therefore conclude that the subacute anti-inflammatory and anti-arthritic activities of the combination are found to be very encouraging. Some compounds, including polyphenols and flavonoids were measured in the extracts forming the combination, which are known to have anti-inflammatory effects [13].Certainly, a clinical study is essential and highly recommended to validate the combination at 250 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana* as an alternative treatment for inflammation.

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**Conflict of interest**

The authors declare that there are no conflicts of interest.

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