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Synergistic anti-inflammatory and analgesic activity of hydroethanolic extracts of *Terminalia macroptera* Guill. & Perr. and *Ximenia americana L.*

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ABSTRACT

Inflammation is the reaction of the immune system to an external or internal aggression of the organism. The symptoms (redness, edema, heat and pain) are secondary to the increase in vascular permeability. The hydroethanolic extract of *Terminalia macroptera* and *Ximenia americana* has anti-inflammatory properties in vitro. The aim was to examine the anti-inflammatory and analgesic activities of extracts alone and in combination. The anti-inflammatory activity was studied using the carragenan-induced paw edema and the type of interactions between the two extracts was determined by the combination index and on the isobologram. Acetic acid-induced torsion and Haffner's tail clamp test were performed to evaluate the analgesic activity. At a dose of 500 mg/kg, *Ximenia americana* and *Terminalia macroptera* extracts showed a percentage of inhibition of 57.02% and 56.53% respectively. The Median-Effective Dose of combination 2 (174.02 mg/kg) was better than that of combination 1 (186.03 mg/kg). The combination index and isobologram indicated a synergistic interaction between the constituents of the two combinations. The extract of *T. macroptera*, *X. americana* and the combination at the dose of 500 mg/kg reduced the number of twists by 46.42; 50.17 and 65.53% respectively. The central analgesic response of the combination was maximal (37.36 ± 8.15 seconds) 60 min after administration. The extracts alone showed anti-inflammatory activity and the combinations showed a synergy of effects. The combination showed peripheral and central analgesic effects.

1. Introduction

Inflammation is the reaction of the immune system to an external or internal aggression of the body. It is manifested by redness, edema, heat and pain. All signs were considered secondary to a primary pathophysiological event, the increase in vascular permeability as a direct consequence of the tissue injury.[1,2] The drugs used against inflammatory phenomena are non-steroidal and steroidal anti-inflammatory drugs. Although these drugs are effective, they are associated with iatrogenic effects such as digestive damage (peptic ulcers, stenosis, perforation) and renal toxicities (acute renal failure, hydrosodic retention).[3,4]

Because of the medicinal iatrogenic of these drugs, plants with anti-inflammatory activity would constitute a more beneficial alternative because of their better tolerance and accessibility.[5] To lead the research of new anti-inflammatory therapeutic agents towards medicinal plants widely used in developing countries by traditional healers for the management of inflammatory diseases would seem imperative.[1]

In Mali, ethnopharmacological surveys have reported throat infections, tuberculosis, vaginitis, inflammation,

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bleeding after menstruation and wound healing as indications of *Ximenia Americana*.[6,7] *Terminalia macroptera* is used against hepatitis, leprosy and tuberculosis, gingivitis, gastritis, colic, fever and the fever and body pains in children.[8]

In addition, pharmacological studies have shown the inhibitory effect of *Ximenia americana* on *M. tuberculosis* H37Rv and the hepato-curative effect of *Terminalia macroptera*.[9,10] The inhibitory activity of proinflammatory enzymes as well as the anti-inflammatory and antioxidant effects of both plants have been demonstrated.[11–13] Given that plant extracts contain several chemical compounds and would have interactions between them or with modern drugs.[14] The objective of this study was to investigate the analgesic, anti-edematous activities and to determine the type of pharmacodynamic interaction between the hydroethanolic extract of *Terminalia macroptera* and *Ximenia americana* on edema.

2. Materials and Methods

2.1. Plant material

The choice of the plant material was focused on the results of previous studies on the inhibitory activity of proinflammatory enzymes and the high content of total polyphenols and flavonoids of the plants.[15,16] It consisted of the lyophilizates of hydroethanolic extract of *Terminalia macroptera* leaves and *Ximenia americana* root bark and combinations based on both extracts.

2.2. Study animals

Male and female NMRI mice weighing between 20 and 30 g were used to perform the pharmacological tests. Wistar rats were used for the toxicity study. The animals were maintained in cages under standard laboratory conditions (12 h light/dark cycle at 25° ± 2°C). They were allowed free access to pellet feed and water. After a 12 h fasting before the tests, the animals were divided into homogeneous groups. In vivo studies were performed in accordance with international guidelines for animal care. All described procedures were reviewed and approved by the Ethics Committee of the University of Sciences, Techniques and Technologies of Bamako (USTTB).

2.3. Acute Toxicity

The acute toxicity of the combination was assessed according to the Organization for Economic Co-operation and Development guideline 423.[17]

2.4. Anti-edematous study

The animals were fasted overnight before administration of the test products. The method described by Anwikar and Bhitre was used for Anti-edematous study.[14] The animals were divided into six groups, composed of positive control, negative control, *Terminalia macroptera*, *Ximenia americana*, combination 1 and combination 2. Groups 1 and 2, negative and positive control (reference product) respectively, received distilled water (10 ml/kg bw) and diclofenac sodium at a dose of 50 mg/kg bw orally. Group 3 made up of subgroups 1 to 5, mice received respectively 100, 200, 300, 400, and 500 mg/kg bw of hydroethanolic extract of *T. macroptera* (TM). Mice in subgroups 1 to 5 of group 4 received 100, 200, 300, 400, and 500 mg/kg bw of the hydroethanolic extract of *X. americana* (XA), respectively. In group 5 or group of the combination 1, consisting of 4 subgroups noted from 1 to 4, mice received 250 mg/kg of TM + 50 mg/kg of XA; 250 mg/kg of TM + 100 mg/kg of XA; 250 mg/kg of TM + 150 mg/kg of XA and 250 mg/kg of TM + 250 mg/kg of XA, respectively. Group 6 where combination 2 group, consisting of subgroup 1, 2 and 3. Mice received 250 mg/kg of XA + 50 mg/kg of TM; 250 mg/kg of XA + 100 mg/kg of TM and 250 mg/kg of XA + 150 mg/kg of TM respectively. At 30 minutes after administration as described above, inflammation was induced in each mouse in the plantar pad of the right hind paw by injection of 50 μl of 1% carrageenan solution in physiological serum. Paw volume was measured 1, 2, 3, and 4 h after carrageenan injection. The percentage inhibition of the increase in paw volume was calculated according to the following formula.[18]

Percentage inhibition = 100 (1 - [A - X/B - Y])

Where, “A” mean paw volume of the treated group after carrageenan injection.

“B” mean volume of the paws of the control group after carrageenan injection.

“X” mean volume of the paws of the treated group before the carrageenan injection.

“Y” mean volume of the paws of the control group before carrageenan injection.

2.5. Quantitation of synergism, additivity or summation and antagonism

A "combination index" (CI) has been designated to quantify synergism, summation and antagonism, as follows:

CI = a/A + b/B

Where, A = ED50 of *Terminalia macroptera*

B = ED50 of *Ximenia americana*

(a, b) = Dose of combination that shows ED50

2.6. Isobolographic analysis of response

Isobolographic analysis was used to characterize drug interactions.[19] The isobologram was constructed by connecting ED_{50} of *X. americana* plotted on the x-axis to the ED_{50} of *T. macroptera* plotted on the y-axis to obtain the additivity line. Combinations are plotted on an isobologram: when this combination is below the additivity line, it shows

synergistic activity and when it is on the additivity line, it shows additive activity. When it is beyond the additivity line, antagonistic activity is indicated.

2.7. Analgesic activity

2.7.1. Acetic acid-induced abdominal writhing test

The analgesic effect of the extracts alone and the combinations was evaluated according to the method previously described.[1,20] After 12 hours fasting, forty-two mice of either sex were randomized into seven groups of six mice each. group 1 and 2 were the negative and positive controls, received distilled water (10 ml/kg bw) and paracetamol (150 ml/kg bw) respectively. Groups 3 and 4 received *T. macroptera* (500 mg/kg bw) and *X. americana* (500 mg/kg bw). Groups 5, 6 and 7 received the combinations as follows: *T. macroptera* (250 mg/kg bw) + *X. amenia* (250 mg/kg bw); *T. macroptera* (250 mg/kg bw) + *X. amenia* (150 mg/kg bw); *X. amenia* (250 mg/kg bw) + *T. macroptera* (150 mg/kg bw) respectively. One hour later, each mouse was injected intraperitoneally with 0.6% acetic acid (10 ml/kg) to induce pain characterized by abdominal contractions or writhing. The writhing reaction per animal was noted five minutes after acetic acid injection and for fifteen minutes. The writhe was indicated by abdominal contraction and stretching of the hind limbs. Analgesic activity was calculated as the percent inhibition of abdominal contraction using the following formula:

$$Percent\ inhibition = \frac{Mean\ of\ negative\ control\ group - mean\ of\ test\ group}{Mean\ of\ negative\ control\ group} \times 100$$

2.7.2. Haffner's Tail Clip method

NMRI mice of either sex with weights between 20-30 g were selected for the study. The mice were randomized into four groups of five each. Group 1 and 2 were the neutral and positive control, the mice received physiological solution (10 ml/kg bw) orally and morphine hydrochloride (5 mg/kg bw) intraperitoneally, 20 minutes before exposure to the noxious stimuli. Mice in group 3 received the combination (250 mg/kg *T. macroptera* + 250 mg/kg *X. americana*) by gavage, 30 min before exposure to the noxious stimuli. After confirmation of the analgesic activity of the Combination, further experiments were conducted to find out its effect on opioid receptors as in group 4, where naloxone hydrochloride (0.4 mg/kg bw) was administered intraperitoneally 30 minutes before the Combination to study its mechanism and site of action. The time taken in seconds to respond clearly will be considered as the reaction time. Thus, the initial time in seconds was recorded individually for all mice in each group before administration of the products. Mice that showed no response within 5 seconds were discarded from the experiment. Reaction time was assessed individually for all mice in each group at 30, 60, 90, and 120 minutes using the Haffner tail clamp method.[21,22]

2.8. Statistical analysis

Experimental data were analyzed using GraphPad Prism 5.03 Software and results are expressed as mean ± SD. One-way variance analysis (ANOVA), followed by Tukey test. Differences were considered statistically significant, very significant and highly significant when $p < 0.05$ (*), < 0.001 (**), < 0.0001 (***) respectively.

3. Results

3.1. Estimation of acute toxicity

A single dose of 2000 mg/kg bw of the combination (1000 mg/kg of *T. macroptera* + 1000 mg/kg of *X. americana*) did not induce any behavioural disturbances in the rats during the first four hours of observation. The behaviour of these rats remained identical to that of rats given distilled water (10 ml/kg bw). The observed effects did not change during the 14 days of follow-up and no mortality was observed. The LD50 was estimated to be greater than 2000 mg/kg.

3.2. Anti-edema effect

The percentage inhibition of extracts alone and both combinations on paw edema is presented in Table 1. The percentage inhibition of paw volume was dose dependent for all products tested. *Ximenia americana* extract at doses of 100 to 500 mg/kg bw showed edema inhibition ranging from 25.52 to 57.02% during the four hours of measurement. At the same doses, *Terminalia macroptera* extract showed inhibition percentages ranging from 21.71 to 56.53%. As for the combinations, with doses of 300, 350, 400 to 500 mg/kg bw of the combination 1, percentage inhibition of edema of 64.86; 67.84; 75.18; 79.66% were observed. Combination 2 at doses of 300, 350, 400 mg/kg bw showed percentage inhibition of 65.25; 69.79; 74.58; Diclofenac (50 mg/kg bw) reduced edema by 83.34%.

3.3. Combination index

The extracts alone and the combinations produced a dose-dependent inhibitory effect on paw edema in mice. The ED_{50} values for the inhibitory effects of the test products are presented in Table 2. In addition, the combination index values were 0.44 and 0.53 for combinations 1 and 2 respectively.

3.4. Isobolographic analysis

The inhibitory effect of combinations 1 and 2 at fixed dose ratios are shown in Figure 1. The isobologram indicates that a synergistic interaction occurred between the constituents of combination 1 and those of combination 2, as can be seen in Figure 1.

Table 1: Percentage inhibition of mouse paw volume

Treatment	Doses (mg/kg)	Edema of the right hind paw	
		A-X	Percentage Inhibition
Negative control		2.64	-
Positive control	50	0.44	83.34
	100	1.96	25.52
	200	1.67	36.55
Ximenia americana	300	1.39	47.36
	400	1.22	53.53
	500	1.13	57.02
	100	2.06	21.71
	200	1.78	32.62
Terminalia macroptera	300	1.49	43.30
	400	1.30	50.83
	500	1.15	56.53
	250 TM + 50 XA	0.93	64.86
Combination 1	250 TM + 100 XA	0.85	67.84
	250 TM + 150 XA	0.65	75.18
	250 TM + 250 XA	0.54	79.66
	250 XA + 50 TM	0.92	65.25
Combination 2	250 XA + 100 TM	0.80	69.79
	250 XA + 150 TM	0.67	74.58

A: volume moyen des pattes du groupe après injection de carraghénane;
X: volume moyen des pattes du même groupe avant injection de carraghénane.

Table 2: ED_{50} of *Ximenia americana* and *Terminalia macroptera* extracts alone and in combination

Plant extract	Equation	ED_{50} (mg/kg)
Ximenia americana	Y = 46,626x - 68,644	350,42
Terminalia macroptera	Y = 50,389x - 80,734	393,09
Combination 1	Y = 70,374x - 109,72	186,03
Combination 2	Y = 65,658x - 97,114	174,02

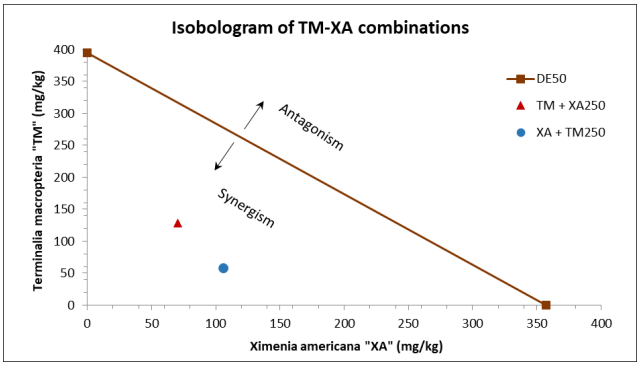

Fig. 1: Isobolograph of interactions between *T. macroptera* and *X. americana* from combination 1 and 2

3.5. Acetic acid-induced abdominal writhing test

The extracts alone and combinations produced dose-dependent antinociceptive activity in the torsion test in mice. The effect of extracts alone, combinations and paracetamol on acetic acid-induced abdominal contractions in mice is presented in Table 3. *T. macroptera* and *X. americana* extracts at 500 mg/kg bw reduced the number of writhing respectively by 46.42; 50.17% compared to the negative control group. The combination (250 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana*) reduced acetic acid-induced pain by 65.53% and paracetamol (150 mg/kg) by 67.58%.

3.6. Effect of T. macroptera, X. americana and combination on pain induced by Haffner's Tail Clip method in mice

Before test products administration, no significant difference was observed between the reaction times of the different groups. The analgesic response was quantified as an increase in the reaction time of mice to dislodge the clip from the artery, showing central analgesic activity. Thirty minutes after administration, the combination produced a highly significant ($P<0.001$) and significant ($P<0.05$) analgesic response compared with neutral control and naloxone + combination group, respectively. Sixty minutes after administration, the combination response was highly significant ($P<0.0001$); at ninety minutes and then one hundred and twenty minutes, this response was significant ($P<0.05$) compared with the neutral control

Table 3: Effect of the extracts alone and the combination on the torsion reflex

Group	Doses (mg/kg)	Number of writhing	Percentage inhibition of writhing (%)
Negative		48.83 ± 3.31	
Paracetamol	150	15.83 ± 2.79***	67.58
T. macroptera	500	26.17 ± 4.67***	46.42
X. americana	500	24.33 ± 3.67***	50.17
T. macroptera + *X. americana*	250 + 250	16.83 ± 3.19***	65.53
T. macroptera + *X. americana*	250 + 150	23.33 ± 3.88***	52.22
T. macroptera + *X. americana*	150 + 250	22.67 ± 4.72***	53.58

When we compare the different groups to the negative control group the difference is highly significant when P< 0.0001 (***).

and the Naloxone + combination group. With regard to morphine, the response was highly significant (P<0.0001) during all measurement phases (Table 4).

4. Discussion

The present study allowed to highlight the non-toxicity, anti-edematous, peripheral and central analgesic properties of the extracts alone and the combinations. LD50 of the combination was estimated to be greater than 2000 mg/kg bw. According to the OECD harmonized classification system, the combination is thus classified in toxicity category 5 which is considered to be non-toxic by the oral route.[17] This estimate is similar to those found on the two plants in the combination by other authors.[12,23]

The carrageenan-induced edema method in the mouse paw is a well-established animal model of acute inflammation to evaluate the anti-inflammatory effect of natural products as well as synthetic chemical compounds.[1] The development of carrageenan-induced edema is biphasic, the first phase is about 1.5 hours after carrageenan injection and is attributed to the release of serotonin, histamine, and bradykinin. The second phase is related to the release of prostaglandins.[24]

The percentage inhibition of paw volume was dose dependent for all products tested. *Ximenia americana* extract at all doses showed a higher edema inhibitory effect than *Terminalia macroptera* extract. Both combinations were more effective in inhibiting paw edema in mice than the extracts alone. Combinations 1 and 2 at a dose of 300 mg/kg reduced edema by 64.86 and 65.25% respectively, against 47.36% reduction for *Ximenia americana* and 43.30% for *Terminalia macroptera*. This efficacy was observed between all doses of the combinations and those of the extracts alone. Anti-inflammatory effect of the extract of *Ximenia americana* and *Terminalia macroptera* has been previously demonstrated by other authors,[13,25] which corroborates the results of this study.

Combinations 1 and 2 reduced edema in a similar manner. The inhibitory effect of combination 2 was greater than the effect of combination 1 at 300 and 350 mg/kg; but the opposite was observed at 400 mg/kg.

The Median-Effective Dose (ED_{50} = 350.42 mg/kg) of *Ximenia americana* was better compared to *Terminalia macroptera* (ED_{50} = 393.09 mg/kg). Both combinations showed lower ED50, in the range of 186.03 mg/kg for combination 1 and 174.02 mg/kg combination 2. ED_{50} characterizes the potency of a product, when ED_{50} is low, reflects a higher potency product.[26] This shows that the combination 2 is higher potency than the combination 1 and very higher potency than the extracts alone. According to several authors, the combination index (CI) is used to categorize pharmacodynamic interactions. When CI is greater than, equal to or less than 1, it can be deduced that an antagonism, a additivity or a synergy of effects, respectively.[27] Our results show CI less than 1 for combinations 1 and 2. It can therefore be deduced that a synergy of effects exists between the components of combinations 1 and 2. Previous studies have specified that the interaction is synergistic on an isobologram, when the combination is below the additivity line [19,24,27]. The isobolographic analysis shown the synergy of effects between the constituents of combination 1 and those of combination 2 (Figure 1). These two methods of classifying pharmacodynamic interactions confirm a synergistic interaction between *X. americana* and *T. macroptera*, the constituents of both combinations.

The acetic acid-induced abdominal writhing method demonstrated the peripheral analgesic effect of the test products with an effect independent of inflammation. The occurrence of abdominal writhing under acetic acid involves the action of local peritoneal receptors, as well as the release of mediators such as prostaglandins.[25,28] Extracts alone, combinations, and paracetamol produced dose-dependent antinociceptive activity. They resulted in a highly significant (P<0.0001) reduction in the number of twists compared with the negative control group. The dose combination (250 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana*) very significantly (P<0.001) reduced edema compared to 500 mg/kg *T. macroptera* and significantly (P<0.05) compared to 500 mg/kg *X. americana*. The analgesic effect of the dose combination (250 mg/kg of *T. macroptera* + 250 mg/kg of *X. americana*) was comparable to the effect of paracetamol (150 mg/kg) and no significant difference was observed.

Table 4: Effect of *T. macroptera, X. americana* and combination on pain induced by Haffner's Tail Clip method in mice

Groups	Reaction time (secondes)				
	Pre drug	Post drug			
	0	30	60	90	120
Negative control	3.02 ± 0.45	3.34 ± 0.69	3.37 ± 0.7	4.29 ± 0.9	3.23 ± 0.52
Morphine	3.1 ± 0.39	41.4 ± 9.34***;###	53.9 ± 5.65***;###	49.41 ± 16.19***;###	29.04 ± 13.37***;###
Combination	3.06 ± 0.45	20.58 ± 6.39**;#	37.36 ± 8.15***;###	24.13 ± 7.92*;#	17.08 ± 6.18*;#
Naloxone + Combination	3.02 ± 0.19	8.05 ± 3.59	5.07 ± 0.82	3.93 ± 0.68	3.47 ± 0.43

When we compare the different groups to the neutral control group or the Naloxone + combination group, the difference is highly significant when P< 0.0001 (***) or (###), highly significant when P< 0.001 (**) or (##), and significant when P< 0.05 (*) or (#) respectively.

Our previous studies have shown inhibitory activity of pro-inflammatory enzymes by these plant extracts. This could explain the peripheral analgesic effect of these extracts and their combinations.[16]

The Haffner's Tail Clip test procedure is based on the observation that morphine-like drugs selectively prolong the reaction time of the reflex to dislodge the tail artery clip in mice, showing central analgesic activity.[21] The central analgesic response of the combination was maximal 60 min after administration with a highly significant lag time (P<0.0001) compared with the negative control. At 120 min the response of the combination was the lowest and showed a significant difference (P< 0.05) as at 90 min compared to the negative group. The morphine response was highly significant (P<0.0001) during all phases of the measurement compared to the negative group. In fact, the morphine response was almost 2 times greater than the response of the combination. Naloxone is a "pure" opioid receptor antagonist.[29,30] We used it as a pretreatment in mice to block opioid receptors. This action caused a significant reduction in the response of the combination. Although slightly higher than the response of the negative group, but no significant difference was observed. This significant reduction in response suggests that the combination would induce its central analgesic response through opioid receptors. The anti-inflammatory and analgesic activity of *X. americana* and *T. macroptera* extract could be attributed to the presence of polyphenol, flavonoids and steroids as revealed by previous studies.[7,15,23]

5. Conclusion

The present study has shown that the hydroethanolic extract of *X. americana*, *T. macroptera* and combinations possess anti-inflammatory, peripheral and central analgesic properties. The data also showed that the two plant extracts combined produced a synergistic effect against acute inflammation. The formulation of these combinations as phytomedicines is an alternative to the clinical treatment of pathologies with inflammatory manifestation.

6. Source of Funding

None.

7. Conflict of Interest

None.

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