Antinociceptive Effect of Ethanolic Extract of *Hybanthus enneaspermus* Leaf in Rats.

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Short title: *Hybanthus enneaspermus and* anti-nociception.

Abstract

Aims : To test the hypothesis that Hybanthus enneaspermus leaf has an antinociceptive effect.

Methodology: Seventy-two male rats were randomly divided in a blinded fashion into 4 groups each for the tail immersion test (n=12 per group) and formalin test (n=6 per group). Group 1 (control) received 0.6 ml of distilled water. Group 2 received 100 mg/kg of acetaminophen (paracetamol). Group 3 and 4 received 500 mg/kg and 1000 mg/kg of ethanolic extract of *Hybanthus enneaspermus* leaf (EEHE) respectively.

Results: In the formalin test, oral administration of 500 mg/kg and 1000 mg/kg EEHE caused inhibitions of 62.48% and 72% in the early phase and 70.54% and 78.63% in the late phase respectively. The 1000 mg/kg dose significantly reduced the paw licking time when compared to the standard drug (acetaminophen) in the formalin test. The 500 mg/kg and 1000 mg/kg doses significantly increased the tail flick latency in a manner comparable to acetaminophen.

Conclusion: This study showed that the leaf has an anti-nociceptive effect.

Keywords: Acetaminophen; Analgesic; Formalin test; *Hybanthus enneaspermus;* Pain; Tail flick test.

1. Introduction

There is a recent upsurge in the use of medicinal plants in herbal remedies for wide range of illnesses in both developing and developed countries of the world [1]. The use of herbal medicine is now recognized as an essential aspect of primary health care [2]. Moreover, most commercial drugs now in the market have their origins in crude use in traditional or folk healing practices [3].

Hybanthus enneaspermus, a traditional medicinal herb belonging to the family violacea is distributed in the tropical and subtropical regions of the world. Its leaf is known among the Yoruba tribe in Nigeria as '*Abiwere'* (meaning leaf that makes delivery painless, trouble-free or fast). Several studies found it to have anti-inflammatory [4,5], hypoglycemic [6], anti-arthritic [4] and antibacterial effects [7]. It is one of the medicinal plants employed by the traditional births attendants in the care of pregnant women. Its leaves are ground with the traditional 'black soap' and used to bathe. Traditional birth attendants claim that the plant invigorates women making the gestational period safe with easy delivery [8,9]. If its local meaning and use are pointers to its actions, we may speculate that *Hybanthus enneaspermus* leaf will have either uterotonic (oxytocic) or analgesic effect or both. Recently, Awobajo et al. [10] demonstrated its uterotonic effect in the myometrial muscle of pregnant rats. There is however a dearth of scientific information on the possible anti-nociceptive effect of oral consumption of the leaf extract. This study was therefore designed to determine whether the ethanol extract of *Hybanthus enneaspermus* leaf will have anti-nociceptive effect when administered to rats.

2. Materials and Methods

2.1. Experimental Animals

Seventy two male albino rats (80-140g) were purchased from the animal house of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology (LAUTECH) Ogbomoso, Nigeria and were acclimated to their new environment. They were fed with standard laboratory diet (Bova Jay Feeds Nig. Ltd, Ogbomoso) with free access to tap water. The rats were kept under condition of uniform humidity and temperature on a 12-h light-dark cycle. Study protocol and animal use were approved, prior to the beginning of the study, by our institutional research and ethical committee and were in accordance with the NIH guide for the care and use of laboratory animals (revised 1978).

2. 2 Selection of Hybanthus enneaspermus and preparation of Ethanolic extract

Hybanthus enneaspermus leaves were bought from a farmer at Oje market in Ibadan, Oyo state, Nigeria and authenticated by Mr. K. A. Adeniji of the Forestry Research Institute of Nigeria (FRIN), Ibadan and a sample specimen voucher number FHI 1008871 was deposited with the FRIN herbarium. They were air dried in a well ventilated and shaded room after which they were ground into a moderately coarse powder (using pestle and mortar). Previous study on the phytochemical analysis of *Hybanthus enneaspermus* has shown that ethanol has stronger extraction capacity, producing number of active constituents responsible for its many biological activities [11]. So, 290g of the powder obtained was extracted with ethanol (70%) using soxhlet apparatus for 48hrs. A semi solid extract (71.57g, 24.6%) was obtained after the elimination of alcohol under reduced pressure. The extract was stored in a refrigerator until used.

2.3 Experimental protocol

Sex differences in pain perception have been reported in numerous studies, with pain thresholds and pain tolerance being lower in females than in males. Previous studies on the estradiol modulation of nociception produced equivocal results, with some demonstrating longer latencies [12,13], while another reported hyperalgesia [14]. Moreover, estrous cycle in female rats has been shown to affect pain perception [15, 16]. Therefore, we investigated the analgesic effect of the extract using male rats.

2. 4 Tail Immersion Test

Forty eight male rats were randomly divided into four groups of twelve rats each as follows: Group 1 rats were pretreated with 0.6ml of distilled water and tested, then treated with 0.6ml of distilled water and retested. Group 2 rats were pretreated with 0.6ml of distilled water and tested, then treated with 100mg/kg of acetaminophen (paracetamol) and retested. Group 3 rats were pretreated with 0.6ml of distilled water and tested, then treated with 500mg/kg of ethanolic extract of *Hybanthus enneaspermus* (EEHE) and retested. Group 4 rats were pretreated with 0.6ml of distilled water and tested, then treated with 1000mg/kg of EEHE and retested.

One hour after each treatment by oral gavage, the tail immersion test was performed as previously described [17]. Animals were handled for 3 min and habituated to the testing room for 1 hour on two occasions before the day of testing and again on the day of testing. The rat was removed from its home cage and gently restrained in a towel, and its tail was immersed in 54°C water. The latency to flick the tail was recorded three times; each time separated by 10 s, and the average of the three measures was calculated. The responses were also analyzed as a repeated measure. All tail flick testing was performed between 9:00 A.M. and 1:00 P.M.

2.5 Formalin Test

Twenty four male rats were randomly assigned into four groups of six rats each as follows: Group I rats (control) were orally treated with 0.6 ml distilled water. Group II rats were orally treated with 100 mg/kg acetaminophen. Group III rats were orally treated with 500 mg/kg EEHE. Group IV rats were orally treated with 1000 mg/kg EEHE.

One hour after each treatment by oral gavage, the formalin test was performed using the method of Hunskar and Hole [18]. Briefly, Animals were habituated to the 30 X 30 X 30-cm transparent Plexiglas observation box for 30 min on two occasions before the day of testing and immediately before testing. Each rat was removed from the observation box and restrained in a towel, and 0.05 ml of 2.5% formalin was injected under the plantar surface of the left hind paw. The rats were placed in the observation box, and the pain behavior within the first 5 minutes of intraplantar formalin injection was recorded as early formalin score, while the pain behavior within 20^{th}- 40^{th} minute of formalin injection was recorded as the late phase. Below the floor of the box, a mirror at a

45° angle facilitated viewing of the injected paw. The behavior was scored as a 2 if the rat licked, bit, or shook the injected paw; as a 1 if the rat elevated the paw from the floor; or as a 0 if any part of the paw other than the tips of the digits was in contact with the box. The score was entered into a computer that recorded the last score entered once every half-second. A mean pain score (a weighted sum of the durations of each behavior) was calculated as the sum of the scores divided by the number of scores in the time period. All formalin testing was performed between 9:00 A.M. and 1:00 P.M.

The percentage inhibition (PI) was calculated using the formula:

$$PI = \frac{\text{Mean Paw licking time (control)} - \text{Mean Paw licking time(test)}}{\text{Mean Paw licking time (Control)}} \times 100$$

2.6 Statistical Analysis

Data were analyzed using Microsoft excel statistical package. All values are expressed as Mean±SEM. While paired t-test was used for the comparison of the before and after administration of the reference drug and the extracts in tail flick test, analysis of variance (ANOVA), followed by a post-hoc Tukey multiple range test for multiple comparisons was used for the change in tail flick latency and the formalin test. Level of significance was set at $P< 0.05$

3. Results

3.1 Tail flick latency test

Table 1 shows the effects of acetaminophen and EEHE (500 mg/kg and 1000 mg/kg) on tail flick latency in rats. Distilled water had no significant effect on the latency period. In contrast to the animals in group 1 which received distilled water at as pretreatment and were retested with distilled water, animals that were retested with acetaminophen (100 mg/kg), 500 mg/kg EEHE or 1000 mg/kg EEHE showed significant increases in tail flick latencies compared to their pre-treatment ($p<.001$) values. Furthermore, high dose of EEHE (1000 mg/kg) significantly ($p<.001$) caused more increase in the tail flick latency than that caused by the reference drug (100 mg/kg of acetaminophen).

3.2 Formalin paw licking test.

Figure 1 shows the effects of acetaminophen and EEHE (500 mg/kg and 1000 mg/kg) on the early phase paw licking time in rats. Animals that received acetaminophen (100 mg/kg), 500 mg/kg EEHE or 1000 mg/kg EEHE showed significant reduction ($p<.001$) in the early phase paw licking time during the early phase compared to control.

Figure 2 shows the effects of acetaminophen and EEHE (500 mg/kg and 1000 mg/kg) on the late phase paw licking time in rats. Animals that received acetaminophen (100 mg/kg), 500 mg/kg EEHE or 1000 mg/kg EEHE showed significant reduction ($p<.001$) in the late phase paw licking time compared to control. Furthermore, high dose of EEHE (1000 mg/kg) significantly ($p<.01$) caused more decrease in the late phase formalin score than that caused by the reference drug (100 mg/kg of acetaminophen).

Table 2 shows the effects of acetaminophen and EEHE (500 mg/Kg and 1000 mg/Kg) on the percentage inhibition of paw licking in rats. While the acetaminophen treated rats showed a higher percentage inhibition in the early phase (68.51%) than in the late phase (63.56%), both the 500 mg/kg and 1000 mg/kg of EEHE treated rats showed a higher percentage inhibition in the late phase (70.55% and 78.64%) than in the early phase (62.68% and 72.86%) respectively.

4. Discussion

The results of our study clearly showed that *Hybanthus Enneaspermus* leaf ethanolic extract (EEHE) possesses antinociceptive activity. In both the tail flick and the formalin test, the extract was effective in reducing nociception in rats. The 500mg/kg of EEHE had anti-nociceptive effect comparable to that of acetaminophen as evident from the insignificant difference in the change in the latency period, and in the early and late phase formalin score in both groups. However, rats that received 1000mg/kg of EEHE had higher latency period, and lower formalin score during the late phase of formalin test compared to those that received acetaminophen. These show that 1000mg/kg of EEHE had more anti-nociceptive effect than acetaminophen.

The greater hypoalgesia in the late phase of the formalin test (70.55% or 78.64%) above that of the early phase (62.68% or 72.86%) in the rats treated with 500mg/kg or 1000mg/kg respectively is of particular interest. Although both early and late phase have nociceptive effect and travel through the same pathways (ie, Aδ and C fibers), the early phase is thought to act by direct chemical stimulation of nociceptors [19], while late phase response acts through inflammation [20] mediated by histamine, serotonin and prostaglandins [21]. The results may reflect reduced activity in chemical sensitivity of afferents, Aδ and C fibers that mediate the second phase response [22]. In the tail-immersion pain test, there was increase in pain tolerance in the EEHE treated rats. However, tail immersion test is a thermal test which travels via the Aδ fiber in which the tolerance may be due to sensitivity of endogenous opiate [23].

Previous studies on the phytochemical screening of *Hybanthus enneaspermus* leaf has shown that it contains saponin, tannin and flavonoids, which are known to have analgesic effect on animals [24,25]. The anti-nociceptive effect exhibited by the *Hybanthus enneaspermus* leaf extract might be due to the synergistic action of its phytochemical components.

5. Conclusion

In conclusion, this study strongly shows that *Hybanthus enneaspermus* leaf has anti-nociceptive effect.

To validate the claim of the traditional birth attendants that *Hybanthus enneaspermus* leaf reduces labour associated pain, further studies are needed on the antinociceptive effect of the extract in non-pregnant and pregnant female rats. Moreover, investigating the particular component(s) with this antinociceptive effect (which is one of the limitations of this study), especially during labour, will help pharmaceutical companies to come out with drugs to alleviate labour-associated pain.

Conflict of interest disclosure

The authors have no conflict of interest to disclose.

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Table 1: Effect of acetaminophen and ethanolic extract of Hybanthus enneaspermus leaf (EEHE) on tail flick latency in rat. Values are expressed as Mean ±S.E.M. (n=12). ***p < 0.001 vs pre-treatment values, ###p < 0.001 vs control values, ΔΔΔ p<0.001 vs acetaminophen group.

	Duration (seconds)		
	Before treatment	After treatment	Change
Control	3.93±0.39	4.04±0.33	0.13±0.08
AMP (100mg/Kg)	2.79 ±0.42	4.33±0.30***	1.56±0.26###
HEE (500mg/Kg)	4.00 ± 0.41	5.70±0.34***	1.69 ±0.35###
HEE (1000mg/Kg)	3.15 ± 0.27	6.26±0.28***	3.11±0.26###, ΔΔΔ

Table 2: Effects of acetaminophen and Ethanolic extract of Hybanthus enneaspermus leaf (EEHE) on the percentage inhibition of paw licking in rats.

	Percentage Inhibition (%)	
	Early phase	Late phase
Acetaminophen (100mg/Kg)	68.51	63.56
EEHE (500mg/Kg)	62.68	70.55
EEHE (1000mg/Kg)	72.86	78.64

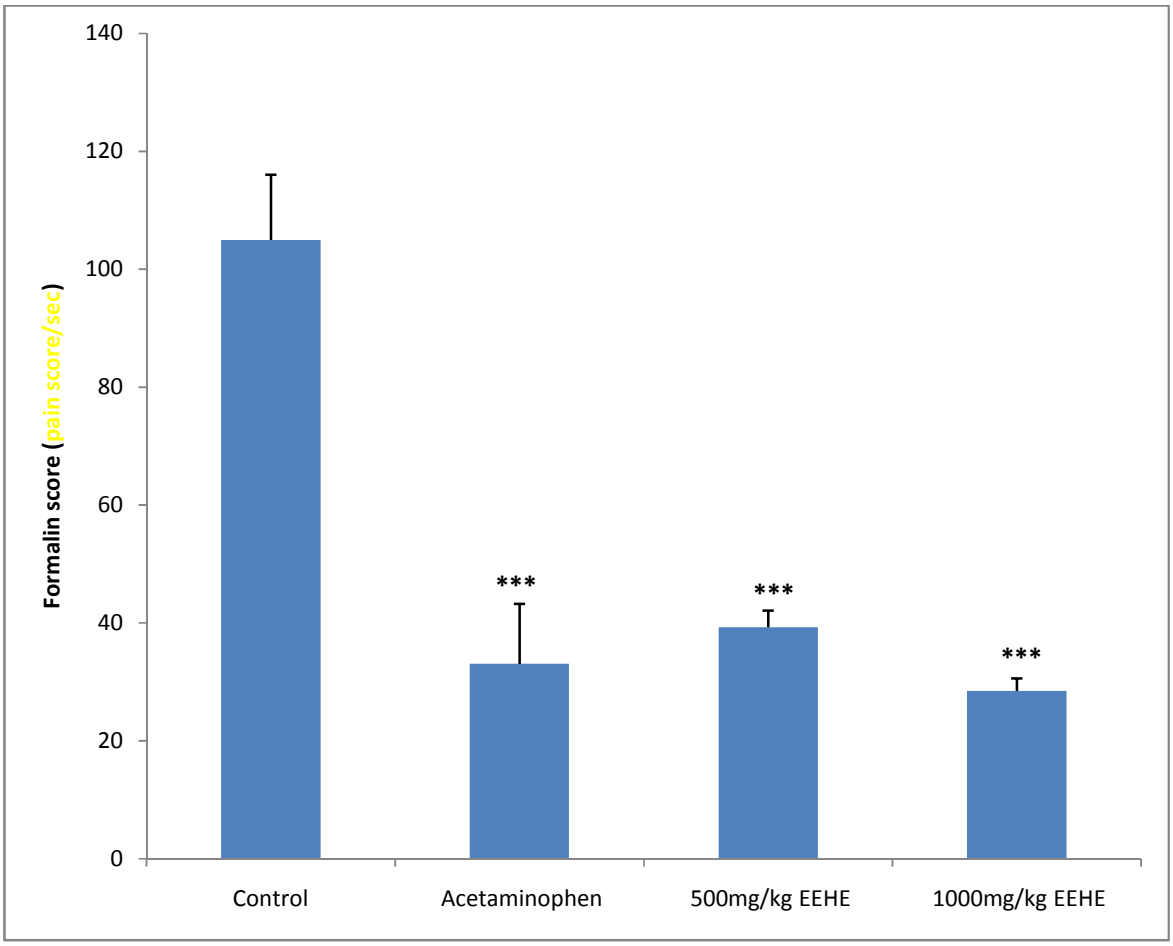

Figure 1: Effects of Acetaminophen and Ethanolic extract of *Hybanthus enneaspermus* (EEHE) on the early phase of formalin score in rats. Values are expressed as Mean±SEM (n=6). ***p<0.001 vs control

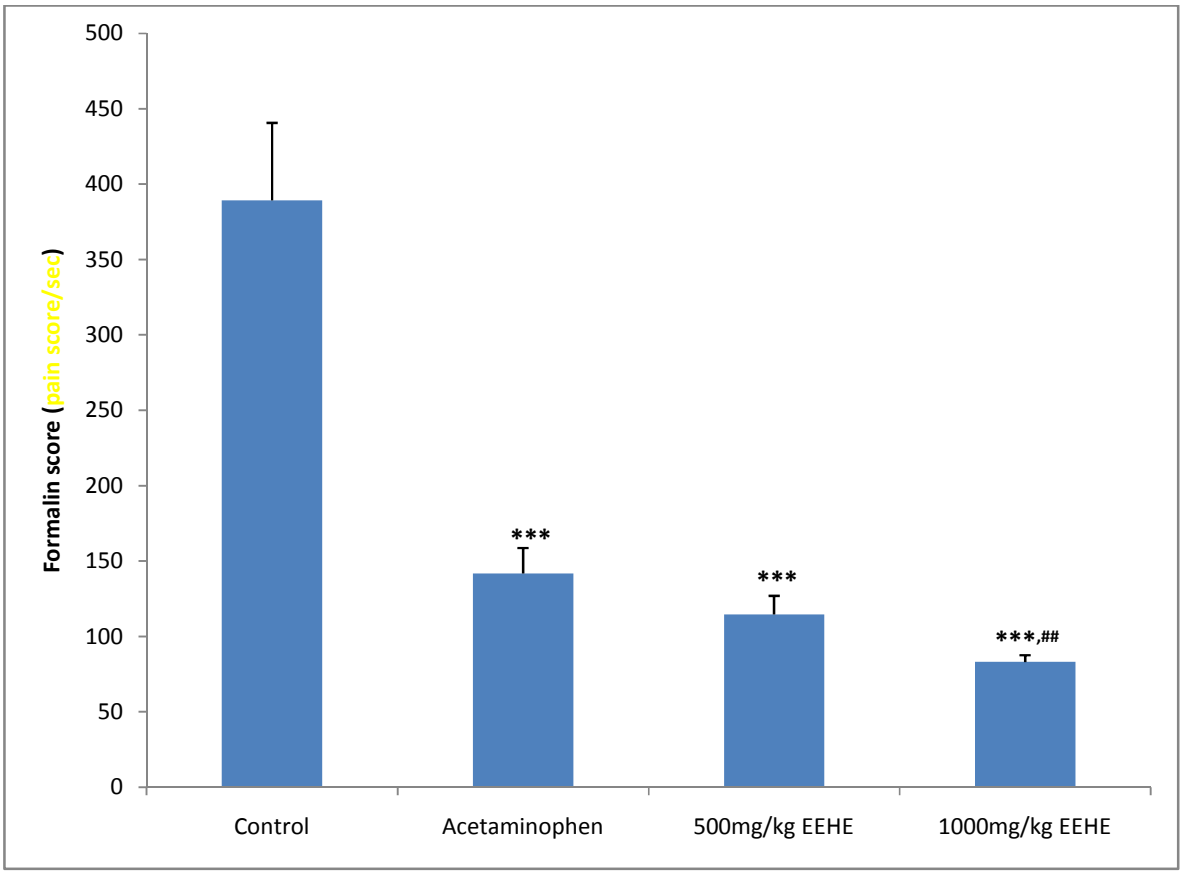

Figure 2: Effects of Acetaminophen and Ethanolic extract of *Hybanthus enneaspermus* (EEHE) on the late phase of formalin score in rats. Values are expressed as Mean±SEM (n=6). ***p<0.001 vs control, ##p<0.01 vs acetaminophen treated group.