Research paper

Antinociceptive ~~effects~~ of ethanolic extract of *Hybanthus enneaspermus* leaf in ~~male albino~~ rats.

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Short title: *Hybanthus enneaspermus and* ~~nociception.~~

Abstract

Aims : *Hybanthus enneaspermus* leaf is one of the medicinal plants employed by the traditional births attendants in the care of pregnant women. While its uterotonic effect has been recently reported, there is scarcity of information on its analgesic effect. We sought to test the hypothesis that it has analgesic effect.

Methodology: Seventy-two male rats were 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 0.6ml of distilled water. Group 2 received 100mg/kg of acetaminophen (paracetamol). Group 3 and 4 received 500mg/kg and 1000mg/kg of ethanolic extract of *Hybanthus enneaspermus* leaf (EEHE) respectively.

Results: In the formalin test, oral administration of 500mg/kg and 1000mg/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 1000mg/kg dose significantly reduced the paw licking time when compared to the standard drug (acetaminophen) in the formalin test. The 500mg/kg and 1000mg/kg doses significantly increased the tail flick latency in a manner comparable to acetamenophen.

Conclusions: This study showed that the leaf has an analgesic effect. ~~Further study should investigate this analgesic effect in pregnant female rats.~~

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

1. Introduction

There has been 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 (Dahanukar, 2000). The use of herbal medicine is now recognized as an essential aspect of primary health care (Onayade et al., 1990). Moreover, most commercial drugs now in the market have their origins in crude use in traditional or folk healing practices (Duke, 1993).

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 short). Several studies found it to have anti-inflammatory (Tripathy et al., 2009; Yoganarasimhan, 2000), hypoglycemic (Awobajo and Olatunji-Bello, 2010), anti-arthritic (Tripathy et al., 2009) and antibacterial effects (Sahoo et al., 2006). 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 (Awobajo et al., 2009; Oyeronke, 2003). 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, Awabajo et al. (2013) demonstrated its uterotonic effects in the myometrial muscle of pregnant rats. There are however dearth of scientific information on the possible analgesic effects of oral consumption of the leaf extract. This study was therefore designed to determine whether the ethanol extract of *Hybanthus enneaspermus* leaf will have analgesic effect when administered to rats.

2. Materials and Methods

2.1. Experimental Animals

Seventy two male albino rats (80-140g) were purchased ~~from the 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 (Anand and Gokulakrishnan, 2012). 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 (Stoffel et al., 2003; Walf and Frye, 2003), while other reported hyperalgesia (Ji et al, 2007). Moreover, estrous cycle in female rats have been shown to affect pain perception (Martinez-Gomez, 1994; Molina et al., 1990). Therefore, we chose to investigate 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 and treated as follows: Group I rats (control) were treated with 0.6 ml of distilled water. Group II rats were treated with 100mg/kg acetaminophen (paracetamol). Group III rats were treated with 500mg/kg of ~~ethanolic extract of *Hybanthus enneaspermus* (~~EEHE~~)~~. Group IV rats were treated with 1000mg/kg of EEHE.

One hour after each treatment by oral gavage, the tail immersion test was performed as previously described (d'Amore et al., 1992). After 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 and treated as follows: Group I rats (control) were orally treated with 0.6ml distilled water. Group II rats were orally treated with 100mg/kg acetaminophen. Group III rats were orally treated with 500mg/kg EEHE. Group IV rats were orally treated with 1000mg/kg EEHE.

One hour after each treatment by oral gavage, the formalin test was performed using the method of (Hunskar and Hole, 1987). 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 0.05ml 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 given are the mean±S.E.M of the variables measured. Significance was assessed by the analysis of variance (ANOVA), followed by a post-hoc Turkey multiple range test for multiple comparisons. P-Values of 0.05 or less were taken as statistically significant.

3. Results

3.1 Tail flick latency test

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

3.2 Formalin paw licking test.

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

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

Table 2 shows the effects of acetaminophen and EEHE (500mg/Kg and 1000mg/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 500mg/kg and 1000mg/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 ethanolic extract of *Hybanthus Enneaspermus* leaf (EEHE) possesses antinociceptive activity. In both the tail flick and the formalin test, the extract was effective in reducing pain perception in rats. While 500mg/kg of EEHE had a comparable analgesic effect to that of the reference analgesic drug (acetaminophen), 1000mg/kg of EEHE had more analgesic effect than acetaminophen. ~~This suggested that the analgesic effect of *Hybanthus Enneaspermus* was dose dependent.~~

The reduction of formalin paw licking response by EEHE in both the early and late phase would suggest that *Hybanthus enneaspermus* has both anti-nociceptive and anti-inflammatory activity as the late phase response has been demonstrated to be due to peripheral inflammatory processes mediated by histamine, serotonin and prostaglandins (Shibata et al., 1989). ~~The greater percentage inhibition of formalin paw licking observed with EEHE than in acetaminophen in the late phase would seem to indicate that *Hybanthus enneaspermus* has a greater anti-inflammatory activity than the reference drug.~~

Previous study 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 (Bittar et al., 2000; Ramaswamy et al., 1985). The analgesic effect exhibited by the ~~*Hybanthus enneaspermus* extract~~ might be due to the synergistic action of its phytochemical components.

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.

5. Conclusion

In conclusion, this study strongly shows that *Hybanthus enneaspermus* has analgesic ~~effect,~~ ~~especially at high dose~~.

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, $^{\Delta\Delta\Delta}$ 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$^{###, \Delta\Delta\Delta}$

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

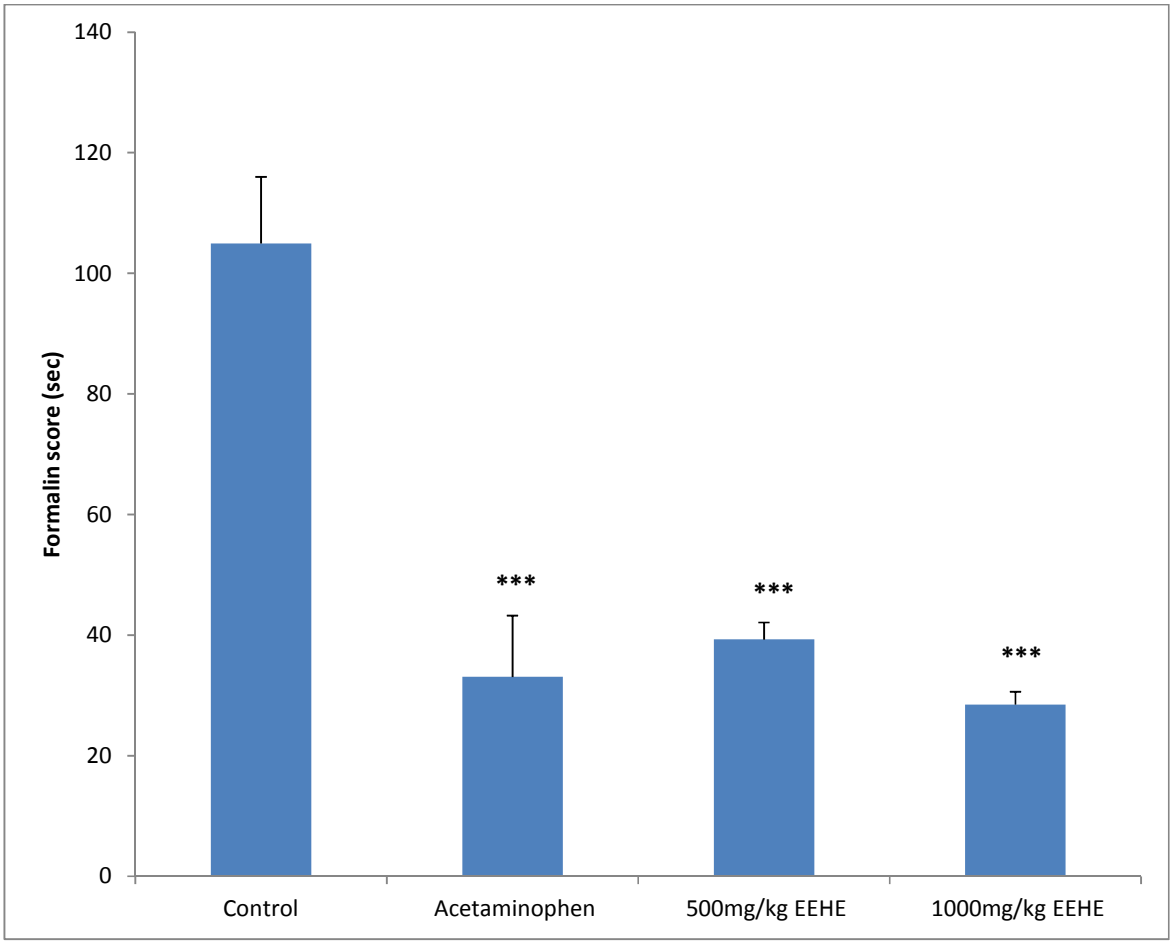

Figure 1: Effects of Acetaminophen and Ethanolic extract of *Hybanthus enneaspermus* (EEHE) on the early phase of formalin score in rats. Values are expressed as Mean±SEM (n=6). ***p<0.001 vs control

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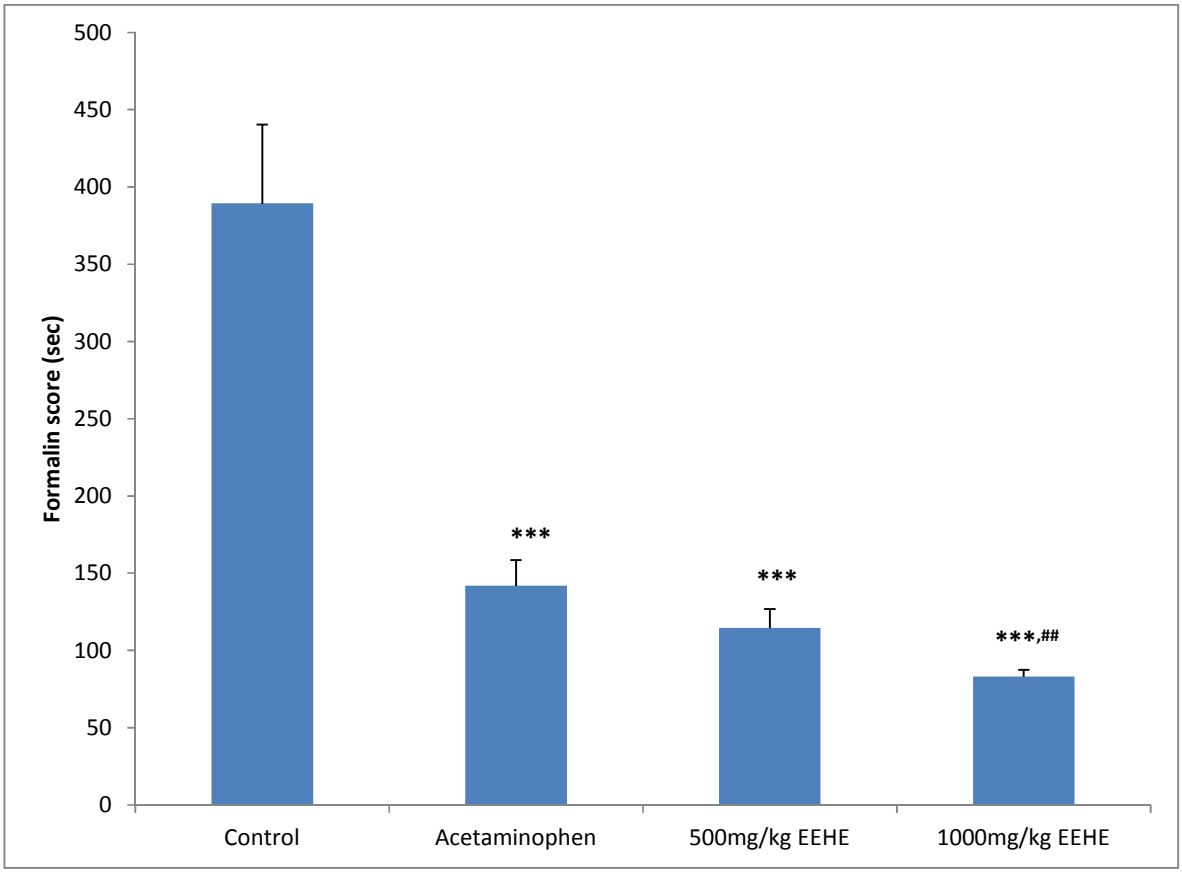

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416 Figure 2: Effects of Acetaminophen and Ethanolic extract of *Hybanthus enneaspermus*
417 (EEHE) on the late phase of formalin score in rats. Values are expressed as Mean±SEM
418 (n=6). ***$p<0.001$ vs control, $^{##}p<0.01$ vs acetaminophen treated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

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