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In-vitro antioxidant activity, biosafety, nociceptive and anti-inflammatory potential of acetone polyherbal (ELNA) extract in mice

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Abstract
ELNA (*Moringa oleifera, Crateva religiosa* and *Curcuma longa*) is a polyherbal formulation comprising of *Moringa oleifera, Crateva religiosa* and *Curcuma longa.* It is used traditionally in the treatment of inflammation, gastro-intestinal infections, hypertension and immune compromised diseases. This study investigate the *in-vitro* anti-oxidant capacity, biosafety, nociceptive and anti-inflammatory potential of polyherbal ELNA acetone extract. *In-vitro* anti-oxidant study was done on the extract using a standard protocol of 1, 1, Diphenyl 2, picrylhydraxyl (DPPH) radical scavenging activity. Toxicological profiling was done using standard methods. Acetic acid, hot plate and egg albumin models were designed for the nociceptive and anti-inflammatory properties of the extract. DPPH scavenging property of the extract showed significant increase at graded concentration. Acute toxicity study of ELNA acetone extract revealed no toxic effect, with absent mortality and less adverse effect (LD_{50}>5000 mg/kg). Subchronic toxicity study of acetone extract administered for 28 days, showed no significant difference (p= 0.05) in organ to body weight values. Haematological indexes indicated no significant different excluding 400 mg/kg that elicited slight significant (p<0.05) increase in the platelet. The results showed that graded doses of acetone extract at (400, 800 and 1200 mg/kg body weight) exhibited significant (p<0.05) decrease in peripheral and central pain also a decrease in the paw edema volume of inflammation. This was achieved in dose dependent manner. The result of this study established a pharmacological evidence for the traditional use of ELNA as an analgesic and anti-inflammatory agent, it also present information on the anti-oxidant properties and toxicity profile of the formulation.

Keywords: biosafety, nociceptive, anti-inflammatory, *In-vitro* antioxidant, polyherbal

1. Introduction
Benefits of herbs as medicine serves as the oldest form of healthcare associated with humanity and cultures throughout history [1]. Early humans acknowledged their reliance on nature for healthy life, since at that time they solely depended on plant the diversity resources for food, clothing, shelter and medicine to cure myriads of ailments. Led by instinct, taste and experience, primitive men and women treated illness by using plants, animal parts and minerals that were not part of their usual diet. Polyherbal preparations are herbal preparation of multiple herbs. Plant formulation and combined extracts of plants are used as drug of choice rather than individual drugs [2], this could be linked to the fact that the individual plants parts making up the polyherbal formulation could act in synergy or have a potentiating effect. Several works have been reported on the effectiveness of polyherbal formulations. In a work on the development and evaluation of analgesic polyherbal formulation containing some indigenous medicinal plants, the formulation was found to have a significant (p<0.05) analgesic activity in a dose dependent manner [3].
Moringa oleifera or Moringa leaves contain several bio-active compounds that exert direct effect on blood pressure thus these can be used for stabilizing blood pressure. Moringa compounds leading to blood pressure lowering effect includes nitrile, mustard oil glycosides and thiocarbamate glycosides present in Moringa leaves [4]. The β-sitosterol present in them is responsible for cholesterol lowering effect [5]. In addition to earlier mentioned bradycardia effect of Moringa leaves, all parts of Moringa are reported with somewhat cardiac and circulatory stimulant activity. Root bark of Moringa contains alkaloid moringinine which acts as cardiac stimulant through its effect on sympathetic nervous system [6].
Crateva religiosa is a much branched deciduous tree belonging to the family Capparidaceae, commonly called as Varuna [7]. Leaves from the plant were utilized by mixing other plants parts recommended by traditional therapists to possess analgesic, antimalarial, antidiarrhoea and antispasmodic properties. It possessed antimitotic activity against *the Candida albicans, Candida tropicalis, Candida krusei, Cryptococus marinus* and *Aspergillus niger*, hepatitis,

edema, ascites, urinary stones and arthritis [8, 9].
Curcuma longa is commonly known as turmeric is a perennial member of the Zingiberaceae family. The rhizome contains active compound curcumin which is responsible for the yellowish colour [10]. Curcumin nature is made up of lipophilic polyphenol insoluble in water but relatively constant in acidic pH of the stomach. Turmeric had been reported to diminish the uptake of cholesterol from the gut and increase the high-density lipids (HDL) cholesterol and decrease low-density lipids (LDL) type. It can also inhibit the peroxidation of serum LDL, which leads to atherosclerotic lesions. Thus, turmeric can prevent coronary problems and heart diseases [10]. This study elicited the biosafety profiling of the polyherbal extract with its therapeutic effect being validated of its nociceptive, anti-inflammatory and ant anti-oxidant properties.

2. Materials and Methods

2.1 Plant Collection

ELNA consist of three herbal leaves (*Moringa oleifera*, *Crateva religiosa* and *Curcuma longa*) and it was obtained from an herbal therapist in Lagos. The plant was identified and authenticated by Dr. O. Timothy a plant Taxonomist in the Department of Plant Biology and Biotechnology University of Benin, Benin City with a voucher number GW-E174.

2.2 Plant Preparation

The various plant was shade dried, pulverized and prepared into extract with a formulation involved the combination of three plants leaves in their dried powdered form at different proportions. It was extracted in acetone solvent system, using Soxhlet Extractor. The filtrate was dried freeze dryer. Sample was stored in a refrigerator for further use.

2.3 DPPH Scavenging Activity

Solution made up of 0.1 mM 1, 1, Diphenyl 2, picrylhydraxyl dissolved and prepared using 98% methanol, and 1.0 ml from the solution was thoroughly mixed in 3.0 ml of acetone extract in methanol comprising of 0.01 – 0.2 mg/ml. Reaction mixture vortexed systematically and left in dark at 37^{o}c for 30 minutes. Absorbance of prepared mixture was recorded using spectroscopy at 517 nm wavelength. Ascorbate was utilized as the standard. Percentage inhibition of DPPH scavenging property was measured in accordance to the stated equation:

$$\% \text{ Inhibition of DPPH} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Where A sample and A control are absorbance of sample and control respectively. Decrease in the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity.

2.4 Experimental Animals

Healthy albino mice weighing between 13-25 g obtained from the Animal House of University of Jos, Plateau State and were kept in the Animal facility of National Institute for Pharmaceutical Research and Development, Idu Industrial Layout, Abuja and kept for about 2 weeks for acclimatization. They were housed in groups of 5 in clean dry cages 34x47x18 cm with soft wood shavings as bedding and maintained in well ventilated animal house with 12 hour light, 12 hour dark cycle. Water and standard pellet feed were given *ad-libitum* for the duration of the study. The procedure was revealed by ethical committee in Life Sciences, University of Benin. The ethical number LS020172 was issued by the committee.

2.5 Acute Toxicity Study

Twelve mice (12) were randomly divided into three groups (n=3) and each group was kept in a separate cage. The first group was given 10 mg/kg of the extract, the second group given with 100 mg/kg of the extract, third group received 1000 mg/kg of the extract and the control group. Four to Twenty four hours later after no death was recorded, phase 2 of the protocol involved three (3) mice administered with 1500, 2900 and 5000 mg/kg of the extract. All were observed after 24 hours all through 14 days using a method described by Lorke [11].

2.6 Sub-chronic Toxicity Study

Two four albino mice of male sex weighing 20 to 25 g were randomly divided into four (4) groups (n=6), each group was put in a separate cage and labelled appropriately. There were administered with graded doses of oral route of the extracts daily for 28. Graded doses of the extract (400, 800 and 1200 mg/kg body weight acetone extract of ELNA and the control group received 0.5 ml/kg body weight of distilled water. Mice were weighed every weekly for the duration of twenty eight days. Animals were anaesthetized and sacrificed, Blood sample was collected via cardiac puncture into EDTA bottle. Organs weight was recorded.

2.7 Acetic acid-induced pain

Twenty five albino mice weighing 13-25 g were randomly selected into groups of five (5) (n=5) after acclimatization. Two methods were adopted to induce pain in mice described by Nakamura *et al.* [12].The writhing response was elicited by intraperitoneal injection of 0.75 % acetic acid at 0.1 ml/10 g body weight. Test substances and control vehicle were intraperitoneally injected into the mice 30 min before acetic acid and the number of writhes was observed and recorded for 15 min beginning from 5 min onset of acetic acid injection.

2.8 Hot plate induced pain

Twenty five animals were randomly divided into five groups (n=5). Including untreated control, 100 mg/kg of aspirin and graded doses of acetone extract at 400, 800 and 1200 mg/kg body weight). Treatment was administered across the groups 16 hr after fasted and were introduced on hot plate (maintained at 55 ^{0}C) after acclimatization. Reaction time is characterized by jumping off or licking of paws) to determine thermal stimuli triggering central pain was noted at 30, 60, 90 and 120 min post extract administration. The mean of the latencies of the animals on the hot plate was determined [13].

2.9 Egg Albumin induced inflammation

The study was investigated via adopted techniques designated by Winter *et al.* [14] Akah and Nwabie [15]. Twenty five Swiss mice were randomly grouped into five (n=5) and treated as follows: Negative control administered with distilled water alone. Reference drug given 10 mg/kg body weight Indomethacin, test groups received graded doses of 400, 800 and 1200 mg/kg of acetone extract of ELNA. Oedema was induced by injecting mice with 0.05 ml of new raw egg albumin in the left hind paw after 30 min of post drug administration. Oedema size was evaluated by using Vernier's Calliper, with results taken at 30 minutes intervals (0, 30, 60, 90, 120 min) after albumin administration.

2.10 Statistical Analysis
Data were analysed using one-way analysis of variances (ANOVA). Group means were compared using turkey multiple comparison test and dunett post-test using a Graph pad prism instant software. Values were represented as mean standard error of mean ± standard error of mean ($P<0.05$) were considered significant.

3. Results
The aqueous and acetone extract showed a significant ($p<0.05$) dose dependent activity, but the aqueous extract had better activity than the acetone extract.

After 48 hours, no deaths or signs of toxicity was observed in the mice treated with the different doses (10, 100, 1000, 1500, 2900, 5000 mg/kg) of both aqueous and acetone extract of ELNA as show in Table 1. Throughout the duration of the experiment, the mice did not show any observable signs of toxicity or morbidity as they looked bright, were feeding well and their faeces looked normal.

Tables 2 shows the changes in body weight of mice treated with different doses (400 mg/kg, 800 mg/kg and 1200 mg/kg) of acetone extract of ELNA. Within each group there was no significant ($p<0.05$) increase in body weight for both extract.

No significant ($p<0.05$) difference of percentage weight gain in heart, lungs, kidneys, liver and spleen of the mice in acetone extracts of ELNA when compared to the control (Tables 3).

The acetone extracts of ELNA significantly ($p<0.05$) decreased the number of acetic acid induced writhes in mice at highest dose of 1200 mg/kg. At lower doses of the extracts showed no significant activity ($p>0.05$). The activity was also noted to be dose dependent (Table 4).

Table 6 revealed that 1200 mg/kg of acetone extract increased latency period significantly ($p<0.05$) at 60 mins. The effect is in dose dependent increase of the latency period for each time with no significant ($p>0.05$).

The results show that acetone extract caused inhibition of egg albumin induced oedema in mice over a period of 3 hrs. These effects were dose and time dependent for all doses but showed significant different ($P<0.05$) at doses of 1200 mg/kg of acetone extract and indomethacin respectively at 120, 150 and 180 min (Tables 7).

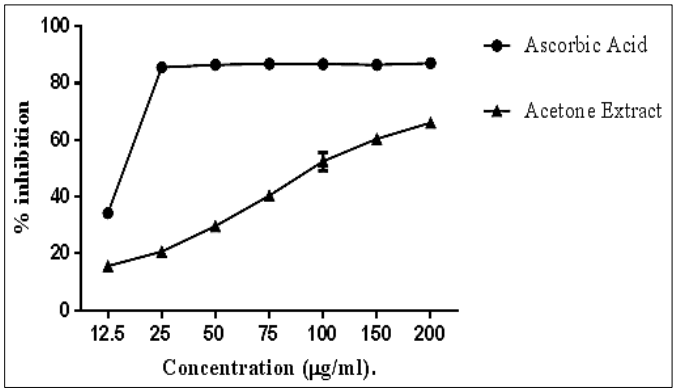

Fig 1: Effect of acetone extract of ELNA on 1, 1, Diphenyl 2, picrylhydraxyl scavenging activity

Table 1: Effect of acute toxicity of acetone extract of ELNA in mice

Treatment	Dose (mg/kg)	No of mortality	No of Mortality and adverse effect
ELNA	10	0/3	Absent
ELNA	100	0/3	Absent
ELNA	1000	0/3	Absent
ELNA	1500	0/1	Absent
ELNA	2900	0/1	Absent
ELNA	5000	0/1	Absent
Control	Dw	0/3	Absent

DW= distilled water

Table 2: Effect of acetone extract of ELNA on body weight of mice.

Treatment/doses mg/kg		Means ±SEM body weight of mice (g)			
		Day 1	Day 10	Day 19	Day 28
Control	Dw	21.00±2.85	20.83±2.88	21.50±2.94	21.67±2.99
ELNA	400	21.50±2.47	19.33±2.31	19.17±2.27	19.17±2.27
ELNA	800	21.17±2.39	19.00±2.07	20.00±1.98	19.83±1.54
ELNA	1200	21.17±2.24	20.33±1.76	20.17±1.87	18.83±1.92

Mean±SEM; *p>0.05*, n=5.

Table 3: Effect of ELNA acetone extract on percentage organ body weight of mice.

Organs	Means±SEM Control Dw	Means±SEM 400 mg/kg ELNA	Means±SEM 800 mg/kg ELNA	Means±SEM 1200 mg/kg ELNA
Heart	0.52±0.61	0.94±0.19	0.53±0.06	0.55±0.04
Lungs	1.09±0.14	0.91±0.05	0.10±0.06	0.95±0.03
Kidney	1.42±0.13	1.57±0.13	1.67±0.23	1.38±0.09
Liver	4.93±0.39	3.72±0.94	5.77±0.23	5.00±0.17
Spleen	0.60±0.04	0.90±0.11	0.65±0.10	0.56±0.07

Mean±SEM; *P>0.05.* n=5.

Table 4: Effect of ELNA acetone extract on the haematological parameters of mice.

Parameters	Means±SEM Control alone	Means±SEM 400 mg/kg ELNA	Means±SEM 800 mg/kg ELNA	Means±SEM 1200 mg/kg ELNA
WBC $\times 10^3/\mu l$	5.15±0.92	9.900±1.42	7.62±2.08	9.24±0.88
RBC $\times 10^6/\mu l$	8.87±0.50	9.320±0.39	8.58±0.44	10.07±0.07
HB g/dl	14.30±0.61	13.220±0.50	12.93±0.61	14.68±0.39

PCV %	47.75±1.71	48.020±1.47	43.37±2.29	52.12±0.67
MCV fl	56.37±5.56	52.200±0.67	50.57±0.44	51.53±0.59
MCH pg	17.03±0.47	15.400±0.18	15.12±0.28	14.07±0.40
MCHC g/dl	29.98±0.47	29.450±0.26	29.88±0.41	28.50±0.76
PLT $\times 10^6/\mu l$	216.80±97.54	907.20±148.10[b]	703.80±246.70	771.70±157.70
LYM %	64.48±8.12	61.800±8.99	85.36±2.79	75.44±4.48
MXD %	7.61±1.81	5.600±1.44	3.04±0.53	6.56±1.87
NEUT %	2852±6.58	18.16±3.05	11.20±2.63	32.40±8.00

n=5; means ±SEM; *$p<0.05$*

Table 5: Effect of acetone and aqueous extract of ELNA on acetic acid induced writhes.

Dose	Doses mg/kg	Mean±SEM number of Writhes
Control	Dw	47.60±1.89
ELNA	400	43.60±2.42
ELNA	800	34.80±4.21
ELNA	1200	29.60±5.71[b]
Aspirin	100	24.80±1.24[c]

Mean±SEM; [b] $p<0.05$; [c] $p<0.01$, n=5. Dw----- distilled water.

Table 6: Effect of ELNA acetone extract on hot plate induced nociceptive in mice.

Time Interval (mins)	Mean±SEM latency period of the animals on the Hot Plate (seconds)				
	Control Dw alone	ELNA 400 mg/kg	ELNA 800 mg/kg	ELNA 1200 mg/kg	Aspirin 100 mg/kg
30	12.15±0.75	13.10±0.60	13.38±2.00	14.90±1.12	12.32±1.24
60	11.60±1.10	9.84±1.56	15.94±1.14	16.24±1.36[b]	15.14±0.75
90	9.76±1.02	9.96±1.24	10.64±1.20	11.68±1.34	13.78±1.27
120	10.32±1.66	9.78±0.70	11.98±0.96	9.78±0.70	9.20±1.70

Mean ± SEM; *$P<0.05$, n=5.

Table 7: Effect of ELNA acetone extract on egg albumin induced right hind paw Inflammation in mice.

Time (mins)	Mean±SEM Control Dw alone	Mean±SEM 400 mg/kg ELNA	Mean±SEM 800 mg/kg ELNA	Mean±SEM 1200 mg/kg ELNA	Mean±SEM 100 mg/kg Indomethacin
30	0.10±0.01	0.13±0.08	0.12±0.01	0.13±0.01	0.12±0.01
60	0.12±0.01	0.11±0.18	0.11±0.02	0.10±0.01	0.08±0.01
90	0.12±0.01	0.10±0.01	0.09±0.01	0.09±0.00	0.08±0.01
120	0.11±0.01	0.09±0.01	0.09±0.00	0.10±0.02[a]	0.06±0.06[c]
150	0.10±0.01	0.08±0.01	0.09±0.01	0.07±0.00[b]	0.04±0.01[c]
180	0.09±0.01	0.08±0.02	0.07±0.01	0.04±0.01[b]	0.01±0.00[c]

Mean±SEM; [a, b, c,] $P<0.05$, n=5.

4. Discussion

4.1 *In-vitro* antioxidant

Low levels antioxidant or inhibitory antioxidant enzymes triggers oxidation and possible impairment or cell death [16]. Free radicals also are responsible for oxidative processes. This study showed 1, 1, Diphenyl 2, picrylhydraxyl (DPPH) scavenging property of acetone extract of ELNA in graded concentration, significantly inhibits oxidative and promote scavenging effect of the extract when compared with ascorbic acid (Figure 1). This study is in line with Siddhuraju and Becker [17] on antioxidant properties of various solvent extracts of total phenolic constituents from three different agro-climatic origins of drumstick tree *Moringa oleifera* leaves. *In-vitro* anti-oxidant evaluation of extract showed significant ($p<0.05$) incline in scavenging DPPH radical using concentration dependent, had a better inhibitory activity. This concurred with the work of Sies [18]; Singh *et al.* [19].

4.2 Acute toxicity

Acute toxicity study could aid the basis for classification and identifying, provide preliminary information on mode of poisonous action in a substance, dose of a novel compound, aid in dose determination in animal studies and determine LD_{50} that provide many indices of potential types of drug activity [20]. Substances with LD_{50} values higher than 5000 mg/kg by oral route are regarded as being safe or practically less toxic. This is in line with the report of Adamu *et al*. [21] with the effect of Aqueous and methanol stem bark extract of *Maerua angolensis*on acute and sub-acute inflammations. Present acute toxicity study, showed absent mortality or signs of toxicity observed after 48 hours all through 14 days of a single and highest doses of acetone extract ELNA (Table 1), this indicated that the extract was safe with wide range of tolerance. Studies from Chainani-Wu [22]; Tripathy *et al.* [23] and Awodele *et al*. [24] carried out on acute toxicity of individual herbs that makes up the herbal formulation with wide safety margin (LD>5000 mg/kg) concurred with present study. These justified the reason why the formulation was also safe. General behaviour and body weight are one of the critical parameters for the evaluation of first signs of toxicity [25].

4.3 Sub-chronic toxicity

In the assessment of the sub-chronic toxicity study of ELNA extract, the body weight of the mice were taken at alternate days throughout 28 days. There was no appreciable weight gain or weight loss, as the body weight showed no significant different ($p<0.05$). The mean percentage organ body weight (heart, lungs, liver, kidneys, spleen) of the mice was not significant ($p>0.05$) across the tested groups of the extract after 28 days (Table 4), though a slight increase in the group administered with the extract. Haematological parameters where relatively stable and no significant increase or decrease

in the extract, with slight increase ($p<0.05$) in 400 mg/kg of platelet count when compared with the control. Awodele *et al*. [24] reported a similar response on toxicological estimation of *Moringa oleifera* aqueous leaf extract.

4.4 Analgesic/Anti-inflammatory study

Herbal therapist outlined ELNA usefulness as a potent anti-oxidant, anti-inflammatory and analgesic agent [26]. Analgesic effect of ELNA acetone extract was demonstrated via acetic acid writhes (peripherally acting) with hot plate (centrally acting) model showed no significant different ($p<0.05$) when compared with the untreated control. Similar results was reported from Gene *et al*. [27] showed *Heterotheca inuloides* anti-inflammatory and analgesic effects. One of the possible mechanisms through which ELNA extract elicited its action via inhibition of the releases prostaglandins PGE2 and PGF2α, triggered by acetic acid. Study from Tanko *et al*. [28] showed similar nociceptive and anti-inflammatory mechanism of action derived from aqueous leaves extract of *Ocimum gratissimum* (Labiatea) in rodents. This is also implicated as a mediator of inflammation [29]. Increase level of prostaglandin within the peritoneal cavity enhanced inflammatory pain via increased capillary permeability [30]. Prostaglandin synthesis is possibly inhibited through peripheral mechanism of pain by ELNA extract as nociceptive and anti-inflammatory effect to reduce the number of writhes rendering analgesic effect when compared with the control (Table 5). The report of Ferdous *et al.* [31] showed the nociceptive activity of aqueous extract of *Ficus racemosa* linn. The acetone extract of ELNA showed significant ($p<0.01$) decreased in writhes at (32.20 and 29.60) respectively specifically at 1200 mg/kg. Generally, acetone extract demonstrated a viable result in dose dependent effect. Hot plate model for assaying nociceptive effects of acetone extract of ELNA on central pain effective in against pain [32]. The acetone extract of ELNA showed a significance ($p<0.05$) decrease at 60 mins for 1200 mg/kg body weight as it aid in reducing mean heat latency period as shown in Table 6. Aspirin displayed analgesic effect with significant decrease in inflammation when compared with untreated control. Based on the effectiveness of the extract on hot plate test, a central mechanism of action could be implicated.

Inflammatory processes involved mediated chemicals such as prostaglandins, histamine and serotonin. Egg albumin-induced oedema from this present study results stimulated of serotonin and histamine release [33]. Anti-inflammatory properties of ELNA acetone extract elicited significant decrease ($p<0.05$) of paw edema at 1200 mg/kg for 120 mins, 150 mins and 180 mins respectively. The extracts under investigation showed significant reduction in inflammation triggered by fresh egg-albumin against the progressive increase in mice paw circumference of the untreated control. Graded doses of the extract suppressed the increase in mice paw oedema in a dose dependent manner two to three hours after inducing inflammation as shown in Table 7. This implies that the extracts will be useful in the management of inflammatory pain. Similar report of Wannang *et al.* [34] whose analgesic and anti-inflammatory activity of the aqueous leaf extract of *Solanum nigrum* Linn (Solanaceae) in Rat.

5. Conclusion

Acetone extract of ELNA possesses a wide range of biosafety with anti-oxidant properties, analgesic and anti-inflammatory, which validated the ethnomedicinal claim by herbal therapist that the formulation is safe with LD_{50} greater than 5000 mg/kg. They were also safe after 28 days of oral administration because there was no residual effect of the prolonged administration.

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