MORPHOLOGIC ASSESSMENT OF INTEGUMENT AND STOMACH OF SPRAGUE-DAWLEY RATS ADMINISTERED *Acalypha wilkesiana* LEAF EXTRACTS VIA MIXED-ROUTES OF EXPOSURE

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ABSTRACT

Background: *Acalypha wilkesiana* family (Euphobiaceae), common name (copperleaf), leaf's juice is robbed on fungal skin infections while oral consumption mitigates stomach disorder claimed by traditionalists. However, there are little or no scientific information in support of these claims.

Aim: This study assessed integument and stomach morphology of rats after a sub-chronic exposure to *A. wilkesiana* leaf extract by mixed-routes of administration.

Materials and Methods: Twenty-five (25) Sprague-Dawley rats, both sexes, mean weight (204.34g) assigned (n=5) labeled (A-E) and polypropylene caged with coconut husks as bedding. Animals were housed in a well-ventilated, neat and hygienic environment, adaptation (14days); temperature (23-25.5°C), humidity (55-60%), and periodicity (12:12hr) while water and feeds were provided regularly. Plant material was identified, authenticated and extracted conventionally. Grouping/administration (A=100mg/kg, B=200mg/kg, C=300mg/kg, D=400mg/kg body weight and E=untreated). Animals were treated via mixed-routes of administration orally (mornings) and subcutaneously (evenings) for 45days at 2days interval. Stool dropping was collected and investigated for fecal occult blood testusing guaiac slide kits. Acute toxicity testing was doneusing anentirely new method.Empirical and physical measurements were conducted before and after extract administration. At the end, all animals were sacrificed by cervical dislocation. Skin and stomach were excised, grossed, fixed and preserved in 10% formalin. Cut tissues (3-5mm) were processed histologically, sectioned (3-5microns), stained (H&E) and examined microscopically. Data were analyzed using IBM SPSS Version 25.0. Groups were compared using ANOVA and presented as Mean ± S.E.M. while p-value≤0.05 were significant.

Results: Morphologically (gross/histology), adverse changes were not observed, though,rats presented with varying degrees of weight losses particularly marked in group E. **Conclusions And Recommendations**: This study suggests that *A. wilkesiana* leaf extract administered via mixed-routes do not have harmful effects on the morphology of target organs, thereby validating the existing safety claims by herbal practitioners.

Keywords: *Acalypha wilkesiana***,** Adverse effects, Copperleaf & Safe dose,

INTRODUCTION

Acalypha wilkesiana belongs to the family of *Euphobiaceae,* with common names as copperleaf, Joseph's coat, fire dragon, and match-me-if-you-can (Oladunmoye, 2006). It is best grown as annual bedding plants or in containers, which is often overwintered indoors, and grows in a single growing season (Stephen *et al.,* 2009). Some of the species are well known in traditional medicine and a few have actually appeared in the homeopathic pharmacopoeia of United States (Ogeyemhe *et al.,* 2019). The leaves of *A. wilkesiana* are popularly used in the north eastern Africa in the treatment of skin infections while the chopped pieces of the leaves are steeped in alcohol and used for stomach ache in southern parts of Nigeria (Iwu, 1993). It has been reported to have dermatological, anti-parasitic, anti-helminthes and anti-bacteria properties (Madziga *et al.,* 2010).

Renewed interest in the use of medicinal plants attributed to cheapness, availability, and accessibility by the populace including high incidences of side effects from synthetic medicines and environmental friendliness have increased the use of herbs (Odigie and Achukwu, 2014). Herbal practitioners claim that natural products are cheaper and more effective than modern medications, which cumulatively brought about revival in the use of herbal products (Madziga *et al.,* 2010; Odigie *et al.,* 2015). There is also a believe that patients in rural communities have a reduced risk of acquiring infectious diseases arising from resistant pathogens than people from urban areas treated with established antibiotics (Odigie *et al.,* 2015). Despite the report from many researchers on *A. wilkesiana* plant, there is insufficient or scares scientific backing to support the safety on stomach and skin (Ogundaini, 2006; Oladunmoye, 2006; Iniaghe *et al*., 2009). Particularly, scientific information relating to safety of stomach and skin morphologies after exposure to A. wilkesiana extracts are lacking; bearing in mind that the administrationof this herb for the acclaimed purposes by herbalists is fondly oral or topical. On the other hand, using mixed treatment regimen will help in promoting proper localization of the plant's extract in the target organs while; examination of the integument and stomach for deleterious effects will aid the establishment and validation of *A. wilkesiana* for treatment of stomach disorders and skin infections. Also, as far as we know, there are no clear cut experimental designs that directly target the integumentary or digestive systems either inanimals or humans, and no established safe dose regime to exclude possible harm to these organs. It is against this backdrop that we assessed morphology (gross and histology) of the integument and stomach of rats for deleterious effects after a sub-chronic exposure to *A. wilkesiana* leaf extract administered via mixed-route of exposure.

MATERIALS AND METHODS

Collection, Identification and Authentication of plant

Sample of fresh *A. wilkesiana* leaf was collected from a private horticultural garden, and was identified and authenticatedat the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Nigeria. A voucher number: UBH_A282 was assigned for referral and cross-reference of possible changes to previous research.

Animal Care, Handling and Ethics

Rats were obtained from the animal house of the Department of Anatomy, University of Benin. They were housed in polypropylene cages, perforated at the sides/ surfaces for adequate aeration, and at the bottom for excretion passages while coconut husks were used as beddings whenever fecal samples drop through the perforated holes. Animals were acclimatized in a serene privately owned animal facility for 14days with standard condition of atmospheric temperature (23-25.5°C), relative humidity (55-60%), and light/dark photoperiod (12:12 hours). Cages were cleaned by replacing bedding husks both morning and evenings. Enough food (Standard Top Feed®) and portable water were adequately provided when needed. Animal study was conducted in compliance with policies outlined in the international best practices for animal experimentation and maintenance of rights of animals updated in the committee's guides for the care and usage of experimental rat's gazette (NRC, 2011).

METHODOLOGY

Preparation and Extraction of Plant material

The leaves of *A. wilkesiana* were thoroughly washed with tap water and air-dried under atmospheric condition for7 days. The dried leaves were repeatedly pulverized into uniform powder using a house hold blender until a pure fine powdered particle was obtained (exhaustive pulverization), which cumulated to 1.3kg. Extraction was by measuring 1kg of the powdered particles with a weighing balance into a sterile conical flask.

After which 1.5L of ethanol was added as extraction solvent with a measuring cylinder and left on the bench for 72hours with intermittent agitations. The solution was filtered by sieving with a white sterile muslin cloth to obtain the filtrate while the residue was air-dried, re-soaked in ethanol and re-filtered repeatedly until an exhaustive extraction was achieved in which the solution becomes colorless after soaking in ethanol. Filtrate was concentrated with a rotatory evaporator to remove residual alcohol and was frozen in a deep freezer at -20^{o}C. Frozen filtrate was freeze dried to obtain a dry powdered form of extract weighing 4.12g and was stored in the refrigerator until it was needed.

Experimental animals

Twenty-five (25) in-bred male and female Sprague-Dawley Rats aged: 11-16weeks, weighing between 196 - 220g were selectively grouped into 5 cages according to body weight and labeled A to E (n=5 rats per cage).

Sub-Acute toxicity test ($L.D_{50}$)

Using the method described by Chinedu *et al.* (2013), another set of rats numbering 16 were selected into 4 groups including a control (n=4). Extrapolated dosage from a similar work was administered in form of 500 mg/kg b.w., 800 mg/kg b.w. and 1200 mg/kg b.w. daily for 4weeks (Odigie and Achukwu, 2015). All rats were monitored for abnormal signs within the initial 4hr after treatment with *A. wilkesiana* leaf extract followed by 24hr intermittent observations.

Empirical measurement

The method described by Ajiboso *et al*.(2007) was used to determine body weight of experimental rats. Individual rat were monitored for daily gain in body weight using digital electronic balance (Gilbertini, Italy). Gain in weight was calculated from the relationship given below: Daily gain in weight= Final day Weight – Initial day Weight, while the mean weight of 204.34g was noted.

Physical measurement

No behavioral signs of acute toxicity were observed in experimental rats either in form of dullness, paw licking, restlessness, anxiety or reduced activities within few hours after administering extracts orally and subcutaneously.

Design and Conduct of experiment

Cage A served as the untreated group otherwise referred to as the control but administered normal saline. Rats in cages B to E were treated with *A. wilkesiana* extract orally and subcutaneously in the following administrative orders:

Group B = 100mg/kg orally (7am) and 100mg/kg subcutaneously (7pm) same day
Group C = 200mg/kg orally (7am) and 200mg/kg subcutaneously (7pm) same day
Group D = 300mg/kg orally (7am) and 300mg/kg subcutaneously (7pm) same day
Group E = 400mg/kg orally (7am) and 400mg/kg subcutaneously (7pm) same day

Note: Extract was administered using both routes of administration as specified above for 45days at an interval of 2days. On the 46th day, all rats were sacrificed by cervical dislocation; stomach and skin were excised, studied and preserved in 10% neutral buffered formalin for tissue processing.

Hidden Blood in Animal Stool

Commercially obtained stool guaiac slide kits (Hemoccult II; Smith Kline Diagnostics, San Jose; califonia, USA) were used to determine the presence or absence of hidden blood in animal fecal sample before, during and after treatment with *A. wilkesiana* leaf extract. An applicator stick (in this case a plastic rubber spoon) was used in collecting few animal fecal droppings from the bedding, and smeared onto one side of the card (usually the left-side). About 3-4 drops of stabilized peroxide reagent were then added to the other side of the card (often on the right-side), which served as a color developer and covered. Controls were added as positive and negative control spots on the test card. Appearance of a blue color within the first 30seconds after interaction between both sides upon covering the test card is indicative of a hidden blood in stool. This test was conducted in the following order: prior to animal treatment, in the course of treatment (i.e 3weeks into the experiment) and after treatment (3 weeks from the previous test).

Processing of Histology Samples

For each of the organs studied, the tissues were fixed for 24hr and were cut at 3-5mm. Thereafter, the tissues were processed in an automatic tissue processor for dehydration, clearing, and impregnation using molten paraffin wax, while embedding was done with the aid of the embedding machine. Sections of the tissues were obtained at 3-5microns using the digital (hertz) rotary microtome (German mode) to produce serial ribbons. Staining of the sections was according to haematoxylin and eosin staining method.The sections were examined using Swift(R) Binocular Microscope with an in-built lighting system and white films with an Olympus photomicroscope® (Opticshot-2; Nikon, Tokyo, Japan). Scale bar using image J protocols was used to calibrate each slide with 0.01mm division while micrograph for x40, x100 and x400 magnification was obtained.

Statistics

Data were analyzed using IBM SPSS Version 25.0. Groups were compared using ANOVA and presented as Mean ± S.E.M. while p-value≤0.05 were significant.

RESULTS

In this experiment, there was no death recorded in the sub-acute toxicity testing phase resulting to a LD_{50} of >1,500mg/kg. Physical assessments of animals revealed that only rats in group E on high dose treatment (400mg/kg oral and subcutaneous) showed mild signs of dullness and reduced activities while empirical measurement indicated that rats in group D and E demonstrated slight weight losses (Table 1). There was no hidden blood detected in animal stool throughout the experimental testing(Table 2). Gross investigation (test and control) did not point at any variation in colour, size and consistency and no evidence of necrosis or lesion in bothtest and control(Plate 1). In the histology analyses, photomicrographs were obtained from control rats and compared to treatment groups, which indicated that there were no alterations between groups treated with mixed treatment plans and the untreated animals (Plate 2).

Table 1: Physical and Empirical Measurements in Experimental Rats

Cages	Dose in mg/kg	Average Weight Before Treatment	Average Weight After Treatment	Physical Weight loss / or gain	Activities / or dullness
A	000	196.44± 1.4	211.66 ± 1.5	↑	-
B	200	198.88± 1.8	192.56 ± 4.7	↓	±
C	400	202.58± 2.3	191.23 ± 3.4	↓	+
D	600	208.62± 1.3	189.10 ± 3.8	↓	++
E	800	218.48± 1.4	189.23 ± 1.1	↓↓	++

Key: Slight increase in weight 218.48± 1.4 (↑), slight weight loss (↓), severe weight loss (↓↓), presence of features (+), intermediate feature (±), marked presence of features (++), absence of features (-)

Table 2: Fecal Occult blood Assessment in Experimental Animals

Animal Groupings in g	Test Before Treatment	Test During Treatment	Test After Treatment
A	Negative	Negative	Negative
B	Negative	Negative	Negative
C	Negative	Negative	Negative
D	Negative	Negative	Negative
E	Negative	Negative	Negative

Note: All animals tested negative to fecal occult blood test prior to experimentation, during and at the end.

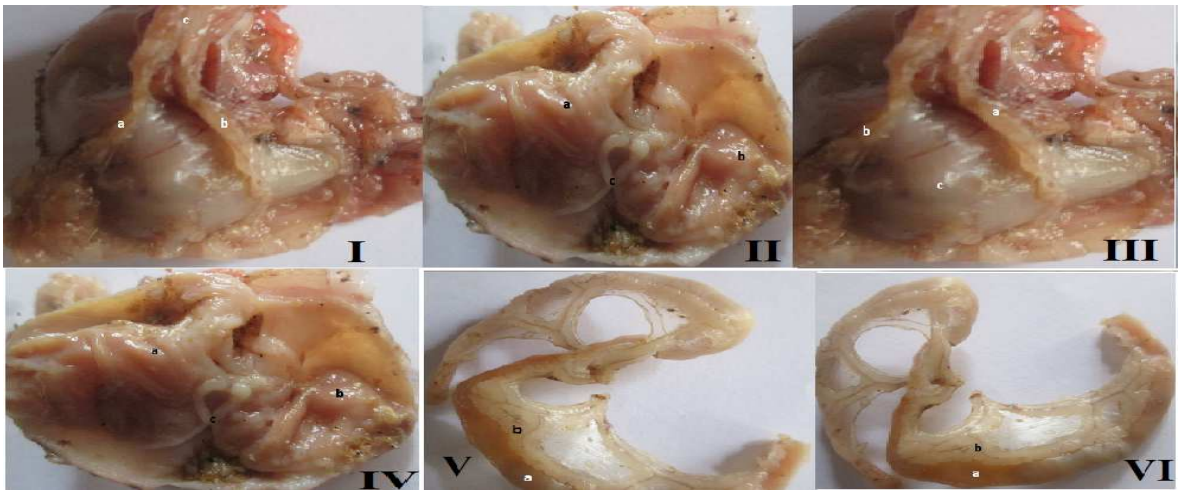

Plate 1: Gross examination of the stomach (**I**) Untreated rats -ventral views with normal pattern showing (a) peritoneal folds, (b) surrounding folds and (c) cardia - conical portion of the stomach (**II**) Control animals -cut surface with (a) greater curvature (b) lesser curvature and (c) *incisura angularis* (**III**) Group E - ventral view treated with 400mg/kg mixed exposure (a) = peritoneal folds, (b) = surrounding folds and (c) the Antrum (**IV**) cut surface of rats stomach in group E (a) = greater curvature, (b) = lesser curvature and (c) = *incisura angularis* (**V**) dorsal view of the skin of untreated rats from thigh region. (a) dermal layer and (b) epidermal layer (**VI**) dorsal view of the skin of rats in group E treated with 400mg/kg of extracts (a) dermal layer and (b) epidermal layer

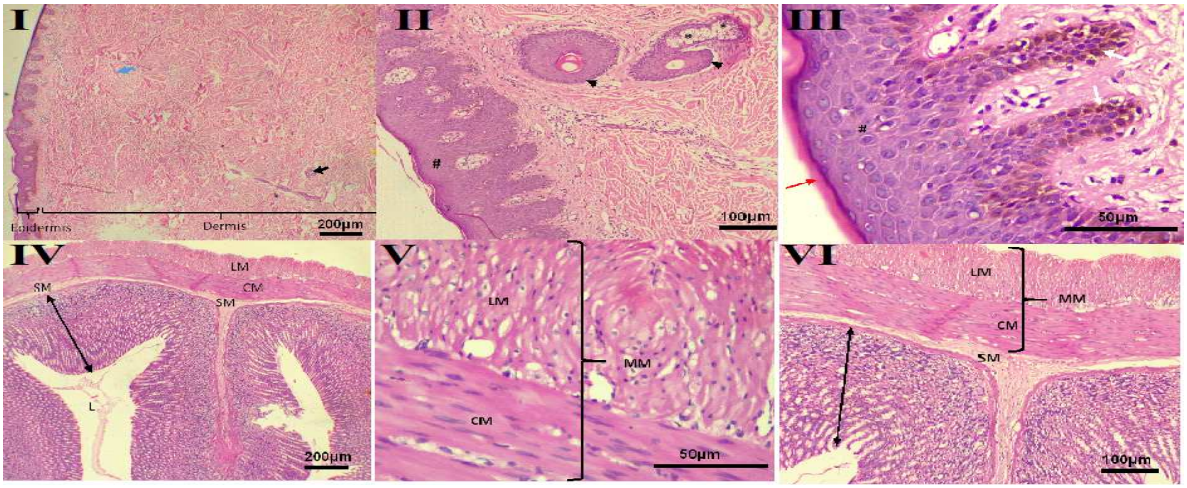

Plate 2: Histology of the skin showed: (**I**) untreated rats in Group A - **Black arrow** = Sweat gland, **Blue arrow**= Arrector pilli muscle, **Thin Red arrow**= Stratum Corneum.(**II**) 300mg/kg treated rats in Group D -**#**represents Stratum spinosum, **Asterisk (*)** = Sebaceous gland and **Arrowhead** = Hair follicle).(III)400mg/kg treated rats in Group E - **Thin Red arrow**= Stratum Corneum, **White arrow** = Melanine Pigment, **#** = Stratum spinosum. **MM** = Muscularis Mucosae (**LM** = longitudinal muscle and **MC** = Circular Muscle. Histology of the stomach: (IV)untreated animals in Group A - **SM** = submucosal, **Double end arrow** ⟷ = Mucosa, **L** = Lumen, **Red arrows** = Gastric pits. (V) 300mg/kg treated rats in Group D - **MM** = Muscularis Mucosae, LM = longitudinal muscle, CM = Circular Muscle. (V) 400mg/kg treated rats in Group E – **MM** = Muscularis Mucosae, LM = longitudinal muscle, CM = Circular Muscle, **SM** = submucosal and **Double end arrow** ⟷ = Mucosa. Sections were in keeping with normal histology of organs after comparing test and control sections respectively.

DISCUSSION

The rate of abuse of medicinal plants (herbal preparations) is alarming in the present day society (Onocha and Olusanya, 2010). At a primary onset of fever and other illnesses, patients result to self-help traditionally, which often leads to macro-anatomical or cellular adverse effects in the long run (Owoyele *et. al.,* 2011). This study thus investigated possible adverse effect of chronic administration of one of such traditional herbs from the plant *A. wilkesiana* with intent to validate it safety as it has been widely claimed by herbal practitioners.Our study did not record any death during toxicity testing and more so there was no variation in the organs of treated animals. It however aligns with the report of Alli *et al.*(2011)in which the leaf of *A. wilkesiana*was reported to be safe for human consumption after examining the photochemical properties as well as some vital organs but excluded the skin and stomach in their investigation. Tobuttress the claims reported by Alli *et al.*(2011), it was further stressed that aqueous leaf extract of *A. wilkesiana* contained tannins and tannic acid in abundance,which is the major secondary metabolites responsible for protecting vital organs from harmful substances (Ikewuchi and Ikewuchi, 2009; Ikewuchi *et al.,* 2011). In other literaturesby Gotep *et al.,* (2009); Madziga *et al.* (2010),it was reported that tannins is well documented to possess anti-microbial properties, which also supports the claim by Haruna *et al.*(2013) that reported the therapeutic use of *A. wilkesiana* leaf for the treatment of stomach upset orchestrated by microbial activities in the stomach. In another development, skin infections have been revealed to originate from one or combinations of fungal interactions with the skin (Owoyele *et. al.,* 2011). Tannins protectiveness and wound healing properties have been reported by Oyewole *et al.* (2011) to be responsible in parts by protecting the integrity of the skin.

In addition, report has it that tannins have soothing relief, which helps in regenerating broken or injured skin with anti-inflammatory and diuretic effects (Okwu and Okwu, 2004). This indication is in tandem with our study in which subcutaneous administration of *A. wilkesiana* leaf extract did not result to potential harm to the skin morphology. Recall that Abraham (2002);revealed that medicaments are well absorbed by the skin when administered subcutaneously. Therefore, this study validates the safety of the skin for topical application of *A. wilkesiana* when applied for the treatment of skin infections. On the other hand, a cross reference of the pharmacological actions with the ethnomedicinal profile of the plant indicated that alkaloids might be responsible for *A. wilkesiana* therapeutic effects for stomach disorders (Borokini and Omotayo, 2012). It is suggested that it may be responsible for the ethnomedicinal usage of *A. wilkesiana* in treating stomach ache, vomiting, intestinal worms, constipation, dysentery and diarrhea (Borokini and Omotayo, 2012). In agreement with Anokwuru *et al.* (2011); Owoyele *et. al.* (2011);Borokini and Omotayo (2012) respectively, *A. wilkesiana* was reportedly used in the treatment of stomach ache, and gastro-intestinal disorders from the days of old. Our study however, validates the safety claims by previous authors earlier mentioned after a mixed method of administration of *A. wilkesiana* leaf extract in this study did not reveal neither gross nor histological effects on stomach of experimental rats. The safety of the gastrointestinal tract and stomach of rats are guaranteed in this study, which supports the report that administration of *A. wilkesiana* leaf extract via oral consumption for gastro-intestinal disorders is safe for human consumption and is not injurious to the stomach (Anokwuru *et al.,* 2011; Owoyele *et. al.,* 2011; Borokini and Omotayo, 2012). The present study strongly corroborates the finding by Onocha*et al.,* (2003); Ezekwesili *et al.,* (2008); Onocha and Olusanya, (2010) in which *A. wilkesiana* leaf extract was reported as safe for use in the treatment of wounds and swellings, including parasitic and helminthic infections.

The fact that there was no detection of hidden blood in the stool samples investigated all through the experimentation strongly suggests that there were no injuries in the digestive system and no intestinal ulcerations resulting from the mixed treatment plan. Particularly, negative occult blood test before experimentation suggests that there were no ulceration or stomach injuries prior to administration of the extract. Histopathology of the skin and stomach in the present investigation are seen to be consistent with normal histology, which thus, support the usefulness of the extract and safety for use. These observations collectively corroborate the report by Owoyele *et al.* (2011) in which *A. wilkesiana* leaf extract was said to be analgesic and anti-inflammatory after exposure to ulcerated animals. Results relating to differences in weight loss or gain in this study have been published (Odigie *et. al.,* 2015), which suggest that *A. wilkesiana* plant extract may support weight loss in humans if fully harnessed.

CONCLUSIONS

This study suggests that the ethanol extract of the leaf of *A. wilkesiana* is relatively fit for traditional treatment of ailments relating to the stomach and integument via oral and subcutaneous deliveries as there were no records of harm to the morphology of the target organs. Our study therefore, validates the existing information on ethno-medicinal claims of *A. wilkesiana* for treatment or management of dermatological and stomach disorders without morphological deleterious effects. More research should be carried out on the toxicity of the plant in order to know the safety and establish a safe dose regimen since the infusion of the leaf is often taken orally.

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AUTHOR'S CONTRIBUTIONS

All authors participated fully in this work to warrant authorship. Dr. B.E Ogeyemheacted on behalf of others to communicate with the editorial team of BJMLS.

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CONFLICT OF INTEREST

No conflict of interest is associated with this manuscript.

AUTHORS DECLARATION

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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