PHYTOCHEMICAL COMPOSITION, BIOACTIVITY AND WOUND HEALING POTENTIAL OF *EUPHORBIA HETEROPHYLLA* (Euphorbiaceae) LEAF EXTRACT

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ABSTRACT

Villagers have traditionally used *E.heterophylla* parts to treat a variety of ailments. The objective of this study was to evaluate the phytoconstituents, bioactivity and wound healing potential of *E.heterophylla* leaf extracts in experimental *Rattus novergicus*.All experiments were conducted following standard procedures. The order of decreasing concentrations of the Phytochemical is alkaloids>cyanide >tannins>flavonoids> saponins. The order of decreasing cytotoxicity is aqueous extract (fresh)> ethanol extract (fresh)> ethanol extract (dry)> aqueous extract (dry). The extracts in the form of an ointment was used for evaluating the wound-healing potential in an excision wound model. The wound-healing property observed in *E.heterophylla* is attributed to the phytoconstituents and the faster process of wound-healing could be a function of either the individual or additive effects of the phytoconstituents.The ethanol extracts exhibited high percentage of wound closure. The prophylactic effect elicited by the extracts in terms of wound contraction and related biochemical parameter strongly support the healing and sealing characteristics of *E.heterophylla* leaf gel in rats following topical administration.

Keywords: *Euphorbia heterophylla*, wound-healing activity, Phytochemical, bioactivity and *Rattus novergicus*

INTRODUCTION

Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds. Many of the indigenous medicinal plants are used as spices and food plants. They also sometimes added to foods meant for pregnant women and nursing mothers for medicinal purposes [1, 2, and 3].

In addition, the use of herbal medicine for the treatment of diseases and infections is as old as mankind. The World Health Organization supports the use traditional medicine provided they are proven to be efficacious and safe [4]. In developing countries, a huge number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have no alternative for primary health care [4].

Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of primary health care cannot be over-emphasized. More so herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly [5]. They are also cheap, easily available and affordable. Many medicinal plants are claimed to be useful for wound healing in the traditional system of medicine. These plant remedies (both single plant and multi-herbal preparations) are used since ancient times even if the mechanisms of action, toxicity and efficacy of very few of them have been

evaluated scientifically. Wound healing is the process of repair that follows injury to the skin and other tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase protein (Collagen) production. Later, the epithelial tissue is regenerated [6].

There is therefore the need to look inwards to search for herbal medicinal plants with the aim of validating the ethno-medicinal use and subsequently the isolation and characterization of compounds which will be added to the potential list of drugs. *Euphorbia* plants are widespread in nature ranging from herbs and shrubs to trees in tropical and temperate regions all over the world [7]. The family *Euphorbiaceae* comprises of 280 genera and 730 species with the largest genus *Euphorbia* having about 1600 species. Generally, they have characteristic milky latex [8], sticky sap; some are co-carcinogenic, severe skin irritation and toxic to livestock and humans [9]. *Euphorbia heterophylla* leaf is used in traditional medical practices as laxative, antigonorrheal, migraine and wart cures. The plant lattices have been used as fish poison, insecticide and ordeal poisons [10,11].In some parts of Kogi State, Nigeria , the leaves are used as anticonvulsant and cough remedy. The leaves *of E.heterophylla* have been reported to contain quercetin [12].Diterpenoids have been reported in the root of *E.heterophylla* [13. The skin irritant, tumor-promoting anti-tumor/anti-cancer and recently anti-HIV activities of *Euphorbia* species have also been reported in *E. heterophylla* leaf [14].

This study investigates the phytoconstituents, cytotoxicity and wound-healing activity of *E.heterophylla* leaf extracts.

MATERIALS AND METHOD

- **Plant material collection and preparation**

Fresh leaves of *E.heterophylla* were collected from Kogi State University Staff quarter, Anyigba, Kogi State, Nigeria. Dirt was removed from the plant by rinsing in clean water .Some portion of the leaves were air-dried for three (3) weeks and pulverized using motorized bender and some portion were used freshly for the extraction.

- **Plant Identification**

The plant sample was identified in the Botany unit of the Department of biological Sciences, Kogi State University, Anyigba, Nigeria as *Euphorbia heterophylla (Euphorbiaceae*)

- **Plant extract preparation**

Cold extraction method was followed. Portions (100g) of the fresh and dry samples were weighed into 1000ml conical flasks and 1000ml of ethanol and or water was added and left for 48 h. The mixtures were filtered under vacuum pressure and the filtrates were concentrated using rotary evaporator and subjected for the various activity studies.

- **Ointment preparation for topical application**

Ethanol and water free extracts of fresh *Euphorbia heterophylla* leaf gel were used for the preparation of ointments for topical application. These ointments for the assessment of excision wound healing activity of the extracts were formulated by using simple ointment BP as base.10% (w/w) ointment was applied where 10g of extracts were incorporated in 100g of simple ointment base BP.0.5g of each of extract ointment and povidone iodine ointment was applied twice daily to treat different groups of animals respectively.

- **Chemicals**

All chemicals and reagents used were of analytical grade and purchased from BDH, Poole,England.

- **Animals**

Wister albino rats (***Rattus novergicus***),(male) of four (4) weeks, weighing between 100 and 150g were obtained from the Department of Biochemistry ,Kogi State University animal house and use for the wound healing study. The animals were housed in standard environmental conditions of temperature ($30\pm1^{o}C$), humidity ($60\pm0.2\%$) and a 12h

light and 12h dark cycle. Rats were fed with standard rodent diet and tap water *ad libitum*.The study was carried out following the guidelines of the principles of laboratory animal care [4].

- **Wound healing Activity**

- **Excision wound model**

The animals were divided into four (4) groups of two (2) rats each.

- Group I served as control
- Group II served as standard treated with povidone iodine ointment topically
- Group III served as test group treated with aqueous extract of *E.heterophylla*
- Group IV served as test group treated with ethanol extract of *E.heterophylla*

The back of animals were shaved and sterilized with 70% ethanol before 7x7mm excision wound was created by a surgical blade from a pre-determined shaved area on the back of each animal [15].The wound was left undressed to the open environment and no local or systemic antimicrobial agents were used . This model was used to monitor the rate of wound contraction. The experimental groups were topically applied with the extracts (ethanol and aqueous) twice daily for consecutive 24 days. The group treated with povidone iodine drug served as a reference. A progressive decrease in the wound area was monitored periodically at every 8^{th} day interval. The wound contractions were measured by a tracing paper on the wounded margin and calculated as percentage reduction in wound area .The actual value was converted into percentage value taking the size of the wound at the time of wounding as 100%.The granulation tissues were removed on the 8^{th} and 16^{th} post wound days and analyzed for protein content (collagen).

- **Estimation of total protein**

Total protein concentration on the regenerated tissues from the healed lesions of wound sample was determined using the method described by [16].

- **Phytochemical Analysis**

The percentage alkaloid in the plant sample was determined by the gravimetric method of [17]. Tannins content was quantified following the method of [18] as described by [19]. The composition of flavonoids was determined by Aluminum chloride colorimetric method as described by [20].The saponins content was estimated by the spectrophotometric method of [19]. Cyanide composition was determined using the method of Wang and Filled method as described by [17].

- **Cytotoxicity Bioassay**

Modified method of [21] was used to determine the inhibitory activity of the extracts on *Artemia salina* in vial bottles. Brine shrimps (*A.salina*) were hatched using brine shrimps eggs in a plastic vessel (500ml), filled with sterile artificial sea water (Prepared using NaCl salt (38g/L) and adjusted to pH 8.5 using 40% NaOH) under constant aeration for 48h .After hatching, active *nauplii* free from egg shells were harvested from brighter portion of the hatching chamber and used for the assay. Ten *nauplii* were drawn through a glass capillary and placed in each vial containing 5ml of brine solution. A portion (50µl) of different concentrations of crude ethanol and aqueous extracts (1000, 500,250, and 125 µg/ml) solution in 1%DMSO-artificial sea water was added into each well(vial bottle) containing 10 newly hatched brine shrimps and then incubated at room temperature for 24h . All samples were repeated in two wells to make the overall tested organisms of 20 for each. The living shrimps were counted under a hand magnifying lens. Same procedure was followed using Potassium dichromate as the reference toxicant (standard).

Plot of % lethality versus log concentration, substituted Y=50 in the resulted linear equation to obtain the X value. The antilog of X was the LC_{50} (concentration of 50% lethality) value [22].

Wound surface microbial load count

The microbial load was determined using the swap method as described by [26]

Statistical analysis

All data were expressed as mean ±SEM and Graph Pad Instat-[DataSet 1.ISD] was applied to determine the significance of the difference at $P<0.05$

RESULTS AND DISCUSSION

The result of the phytochemical analysis is as presented in Table 4 and it revealed the presence of medicinally active constituents. The quantitative estimation of the percentage crude yields of chemical constituents of the plant studied showed that the leaf of the plant *(E.heterophylla*) is rich to some extent in alkaloids ,flavonoids ,saponins, and tannins .They were known to show medicinal activity as well as exhibiting physiological activity [23].*E.heterophylla* contains high amounts of alkaloids and low saponins content. The order of decreasing concentration of the Phytochemical is Alkaloids >Cyanide >Tannins > Flavonoids >Saponins. The result of the wound healing activity of *E.heterophylla* extracts are presented in table 1. The extracts' healing activity is comparable to the reference standard (Povidone iodine). Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissues as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema and new small blood vessels.

The ethanol extract of *E.heterophylla* demonstrated a significant increase in protein and wound closure. Any one of the phytochemical constituents present in *E.heterophylla* may be responsible for the wound-healing activity. Recent studies with other plant extracts have shown that phytochemical constituents like flavonoids [24] are known to promote wound healing process mainly due to their astringent and antimicrobial properties, which appears to be responsible for wound contraction and increased rate of epithelization.

The wound-healing property of *E.heterophylla* may be attributed to the phytoconstituents present in the plant and the faster process of wound healing could be a function of either the individual or the additive effects of the phytoconstituents. Tannins for instance ranked third in quantity among the Phytochemical estimated in *E.heterophylla*. Tannins are astringent and antimicrobial in property , hence it can be inferred that the wound-healing activity of the leaf extract of *E.heterophylla* observed is due partly to its tannin and flavonoids contents, which seems to be responsible for wound contraction and increased rate of epithelization.

The observed increase in protein (collagen) (table 2), an important constituent of extracellular matrix in the treated animals confirmed that the extracts had positive effects towards cellular proliferation, granulation tissue formation and epithelization. The increase in protein content in the treated group is predominantly due to enhanced collagen synthesis in the *E.heterophylla* gel extract treated groups.

The capacity to form supramolecular aggregates in extracellular spaces is one of the important characteristics of molecules belonging to the collagen family of proteins [25]. The hydrophilic nature of the collagen attributed by its molecular structure characterized by high content of diamino-dicarboxylic amino acids and carbohydrate moieties provides a surface geometry very suitable for cell adhesion. In addition to the surface properties, the presence of the glycoprotein like fibronectin on the surface of the cells promotes attraction of fibrogenic cells to collagen. These fibronectin molecules have a high affinity for collagen and link specifically with definite regions on the collagen surface.

The importance of collagen in wound healing has been appreciated for a long time for the simple reason that the ultimate result of most repairs in the higher vertebrate is the formation of scar tissue composed of collagenous fibers.

As shown in table 2, there was a sharp increase in protein content from day 8 to 16.The untreated group showed a slow gradual increase in protein content. The present study has demonstrated that an ethanol extract of *E.heterophylla* has properties that render it capable of promoting wound-healing activity compared with standard treatment controls.

E.heterophylla leaf is commonly used traditionally for the management of convulsion and treatment of cough. In this study also we undertook bio-safety screening and the result is as shown in table 3.The order of decreasing toxicity when compared to the standard (potassium dichromate) is aqueous extract (fresh)> ethanol extract (fresh)> ethanol extract (dry)> aqueous extract (dry).The fresh leaf extracts of *E.heterophylla* are more toxic than the dried leaf extract of both ethanol and water. The toxicity of the extracts are dose dependent and is advisable to use lower doses of the plant parts and prolonged usage should be avoided .Dose formulation is beyond the scope of this research.

Table 5 shows the result of antimicrobial characteristics of *E.heterophylla*. The extracts are potent on microorganisms reducing their population on the wound surface. On day 8 the wound surface was still wet and attracts microorganisms but as the healing progresses there was decrease in microbial population. The aqueous extract gel was more potent than the ethanol extract.

Table 5: Wound surface microbial load count (cfu/ml)

Group/treatment	**Microorganism count after days**		
	8	**16**	**24**
Wound control	84	92	47
Povidone iodine	88	88	65
Aqueous extract	44	32	28
Ethanol extract	60	56	43

Figure 1 a-j shows the healing pattern or the rate of wound contraction in untreated and treated groups. The ethanol extract showed a better healing activity and it is comparable to the reference standard (Povidone iodine). In general, the wound closure rate was rapid in treated groups when compared with control rats. A well-advanced organization of granulation tissue was noticed in leaf gel treated animals on day 16 and complete healing formation of scar was found in rats after 24 days treatment with *E.heterophylla*. Topical application of *E.heterophylla* leaf gel extract at the wound site resulted in significant wound healing which may be due to its angiogenic and mitogenic potential. The enhanced rate of wound contraction and reduction in healing time in treated rats when compared with control might be due to enhanced epithelization aciliated by *E .heterophylla* gel compositions.

CONCLUSION

The aqueous and ethanol extracts showed significant wound healing activity when topically administered on rats. These results offer pharmacological evidence on the traditional use of *E.heterophylla* leaf gel for healing wounds. Further studies are needed to better assess the potential value of *E. heterophylla* extracts as wound healing agents.

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Table 1: Effect of *E.heterophylla* extracts on wound healing in *Rattus novergicus*

Groups /treatment	Percentage closure of excision wound area after days		
	8	16	24
Wound control	42.86±0.007	50.13±0.125	*
Povidone iodine	64.28±0.010	85.73±0.020	**
Aqueous extract	50.06±0.060	71.45±0.020	**
Ethanol extract	57.17±0.015	78.56±0.010	**

Each value represents mean± S.E.M, * 99.9% closure, **100% closure, P<0.05 when compare to control.

Table 2: Effect of the *E.heterophylla* leaf extracts on wound protein concentration in *Rattus novergicus*

Groups/treatment	**% protein concentrations after days**		
	8	16	24
Wound control	17.63±0.76[a]	20.10±0.25	ND
Povidone iodine	18.13±0.17[a]	30.17±0.13	ND
Aqueous extract	31.03±0.07	35.05±0.07	ND
Ethanol extract	34.00±0.26	37.02±0.23	ND

Each value represents Mean±S.D, Mean with the same alphabet in the column are not statistically significant(P<0.05), ND =Not determined –wound completely closed.

Table 3: Inhibitory effect on brine shrimps of *E.heterophylla* leaf extracts

Sample	**concentration (µg/ml)**	**Log Concentration**	**% Lethality**	**LC_{50} (µg/ml)**
Ethanol extract	1000	3.0000	80	198.40[a]
(Fresh leaf)	500	2.6990	60	
	250	2.3979	60	
	125	2.0969	40	
Ethanol extract	1000	3.0000	80	353.52[b]
(Dried leaf)	500	2.6990	40	
	250	2.3979	40	
	125	2.0969	40	
Aqueous extract	1000	3.0000	60	353.78[c]
(Dried leaf)	500	2.6990	60	
	250	2.3979	40	
	125	2.0969	40	
Aqueous extract	1000	3.0000	100	125.05[d]
(Fresh leaf)	500	2.6990	100	
	250	2.3979	80	
	125	2.0969	40	
Potassium	1000	3.0000	100	91.84[e]
Dichromate	500	2.6990	100	
	250	2.3979	100	
	125	2.0969	40	

(a)Linear equation: y=39.86X-41.58, (b) Linear equation: y=39.86X-51.58, (c) Linear equation: y=26.56X-17.72, (d) Linear equation:y=66.43X-89.31 (e) Linear equation:y=59.79X-67.37

Table 4: Quantitative analysis of the phytochemicals of *E.heterophylla*

Plant parts	**Phytochemical composition**				
	CN(µg/g)	%Alkaloids	%Saponins	%Flavonoids	% Tannins
Fresh sample	2.9±0.26	3.72±0.58[a]	0.17±0.01	0.33±0.02[a]	0.81±0.02[a]
Dried sample	2.8±0.04[a]	8.92±0.	0.35±0.01	0.80±0.01[c]	1.24±0.20[c]
Ethanol extract (Fresh sample)	1.66±0.14	5.03±0.05[bc]	0.61±0.02	2.77±0.15	1.31±0.20
Ethanol extract (Dried sample)	0.58±0.04	5.45±0.49[a]	0.45±0.02	0.47±0.05[abc]	0.67±0.02[a]
Aqueous extract (Fresh sample)	3.38±0.03	2.00±0.05[bc]	0.12±0.02	0.40±0.01[abc]	1.47±0.10[bc]
Aqueous extract (Dried sample)	4.00±0.21	7.10±0.90	0.21±0.01	0.30±0.01[bd]	2.09±0.02

Mean values represents Mean± S.D of three determinations. The mean with same alphabet in the column are not significantly different from each other (P<0.05)

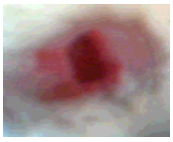

Fig 1(a)fresh wound

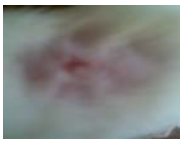

Fig 1(b) untreated after 8 days

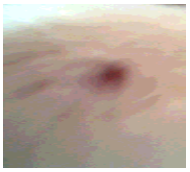

Fig 1(c) Day 8 treatment with aqueous extract

Fig1(d) Day 8 treatment with ethanol extract

Fig 1(e) Day 16 treatment with aqueous extract

Fig 1(f) Day 16 treatment with ethanol extract

Fig 1(g) Day 24 treatment with ethanol extract

Fig 1(h)Day 24 treatment with aqueous extract

Fig 1(i) Day 24 treatment with povidone iodne

Fig 1(j) Day 24 untreated wound

Fig .1:Effect of *E.heterophylla* gel on wound healing

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