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FORMULATION AND IN VITRO EVALUATION OF SOLID DISPERSIONS OF METHANOLIC EXTRACT OF *VERNONIA AMYGDALINA*- PEG-4000: A WOUND HEALING STUDY

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ABSTRACT

Objective: Preparation containing *V. amygdalina*-PEG solid dispersion SDs was evaluated for wound-healing potential on different experimental models of wounds in rats. **Methods:** *Vernonia amygdalina* solid dispersions SDs containing varying concentrations 0:1, 1:0 1:1, 1:3 and 3:1, of *Vernonia amygdalina:* PEG 4000 were prepared using the fusion -solvent evaporation method. Wound contraction ability in excision wound model was measured at different time intervals and study was continued until wound is completely healed. The effects of the preparation on the activities of liver were similarly assessed. **Results:** Tensile strength was measured in 9th -day-old incision wound. Preparation (d) containing 1:3 of PEG : *V. amygdalina* showed statistically significant response, in terms of wound contracting ability, wound closure time, period of epithelization, tensile strength of the wound, when compared with the individual components and the control group (negative control), the results were comparable to those of a commercial neomycin formulation (positive control). The liver parameters and hematological studies did not show much variation to that of the control. **Conclusion:** It is believe that SDs of this extracted could be formulate into pharmaceutical dosage form and used as an alternative in wound healing.

KEYWORDS: Soilid dispersion, PEG-4000, wound, V. amygdalina.

INTRODUCTION

Healing of wounds starts from the moment of injury and can continue for varying periods of time depending on the extent of wound.[1] Wound healing is a complex process involving a series of continuous phase by mechanical and chemical injuries and tissues release some factors at the wounding sites. This event occurs by an interconnected process of regeneration dermal and epidermal tissues that involve the migration, proliferation, adhesion and differentiation of cells.[2] Wound healing is influenced by local and systemic factors of collagen fibers by reducing neovascularization and epithelialization rate under the regulation of special mediator that secreted blood platelets, macrophage, lymphocyte and so on.[3] It is generally composed of three particularly phases; inflammation, proliferation, and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue.[4] Wound healing is a complex series of interrelated events that are mediated through the phases by a wide range of chemically co-ordinate cellular processes as well as hormonal influences.[5] Several antibiotic drugs have been used in the treatment of various types of wound, and many have also been formulated such as ointments and wound dressings used in the treatment of severe skin wounds or ulcers including bedsores and burn wounds.[6] Wounds recover through several disease stages have also been evaluated. In bedsores and burn wounds, the wound stages are often divided into an infectious period, necrosis and agglutination period, proliferation period and epidermis formation period.[7] Generally, formulations are selected based on the disease stage and the causes of the wound. There are increasing resistance to some of the orthodox antibiotics used in wound healing, this were associated to the high level of bacteria resistance that has ravaged our health system due to adulteration, poor drug combination and inactivation of the antibiotic by some enzyme producing bacteria.[8] The practical approach to this problem is to implore of the most popular traditional approach where many herbal product are used locally by traditional herbal practice. Plants and plant products present some hope to scientists, serving as an alternative avenue to discovery from the current mainstream approach of attempting to find solution to disease that has proved very resistance to western drugs for specific health problems.[9] Numerous medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and are still widely used and have considerable importance in international trade.[10] One of the herbs that have great potential in wound healing is *Vernonia amygdalina*. *V. amygdalina* (family, compositae) is a valuable shrub that is widespread in East and West Africa.[11] In Nigeria, it is commonly known as “bitter leaf” because the leaves and stem have a bitter taste when chewed. This is in addition to its leaves being used as a

popular vegetable for soups particularly among the Igbos of Southern Nigeria. Its use for fever, laxative, pile (haemorrhoids) and gastro-intestinal troubles have been reported.[12-3] Its anti-thrombic and anticoagulant properties have also been evaluated.[14] Alcoholic's extracts have been known to possess anti-diabetics property.[15] The raw leaves and it methanolic extract have shown wound healing properties has been used in wound healing.[16-17] Polyethylene glycol (PEG) 4000 is a biodegradable and non-toxic macromolecule that has been used in drugs delivery, it has been mixed with honey and pollen extract for treating test lesions in milking cows' and for preventing peritoneal adhesions by promoting non-adherent healing.[18]

A review of the literature revealed that the wound-healing property of this plant has not been subjected to scientific evaluation. It is for this reason, that this study is primarily designed to formulate solid dispersion of the *V. amygdalina* methanolic extract-PEG 4000 and evaluate it wound healing potential in experimental rats.

MATERIALS AND METHODS

Materials

Methanol was purchased from Lavans Chemicals Ltd, Enugu, Nigeria. PEG-4000 was purchased (Union Carbide, Danbury, CT USA). Double distilled water was used throughout the study and all the other chemicals used were of analytical grade and were used without further purification.

Extraction of the V. amygdalina with methanol

Fresh leaves of *V. amygdalina* were obtained from Pharmacognosy garden, university of Nigeria, Nsukka, and identified by Mr. Ozioko in BDCP centre Nsukka, the Voucher specimen was deposited at the Herbarium of the department. The leaves were washed with distilled water and shade-dried for 4 to7 days and was then finely powdered using electrical blender. About 255 g of powder was subjected to soxhlet extraction with 75 % methanol (2.5 liters) in a conical flask for about 48 h. The extract was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and concentrated in vacuum under reduced pressure using a rotary flash evaporator (Sigma – Aldrich, USA) and dried in a desiccator over fused calcium chloride. The semisolid extract (14.5 % yields) was packed into a close tight container and use for further study.

Phytochemical screening of the extract

The methanolic extract was phytochemically screened for the following components; carbohydrates, cardiac and cyanogenic glycosides, tannins, saponin, anthraquinones, flavonoids and alkaloids according to the established standard. [19]

Preparation of solid dispersions SDs of *V. amygdalina*- PEG

The solid dispersions SDs were prepared in the weight ratio; 0:1, 1:0 1:1, 1:3 and 3:1, of (*V. amygdalina*: PEG-4000) by fusion-solvent method and labeled as A-E. Briefly, the required amount of PEG 4000 was melted in a beaker on a water bath maintained at 60–65°C. The appropriate amount of V. amygdalina extract mixed in methanol was added to the molten PEG- 4000 and stirred thoroughly with a glass rod for 10 min. The fused mass was solidified immediately in a freezing mixture of ice and sodium chloride under constant stirring. The mass was pulverized using an End Runner Mill (RS145 Germany) and sifted through 100-mesh sieve and stored in a desiccator over fused calcium chloride and used for further study.

Animal protocol

Wistar albino strain rats of either sex weighing 180-220 g were procured from the Biochemistry Department, University of Nigeria, Nsukka and were maintained at standard housing conditions with 12 h light. The animals were fed with a commercial diet (Feeds BC, Nsukka, Nigeria) and water ad libitum during the experiment. The study was permitted by the Departmental animal research committee ethics, University of Nigeria Nsukka.

Wound healing study

The excision and incision wound models were used to evaluate the effectiveness of the solid dispersion formulated on wound-healing activity. In this study, the guideline on the use of animal was approved by the Ethical Committee of Department of Pharmaceutics, University of Nigeria Nsukka, Nigeria.

Excision wound

The wound site was prepared following the excision wound model.[20] The animals were anaesthetized with ketamin® (Rotex Ltd – Indian), at a dose of 1 ml/kg body weight, immediately the drug take it effect, a clean sterile surgical blade of size (NO. 20) was used to shaved the hairs on the skin of both thighs. A circle of diameter 20 mm was gently marked on the shaved portion of the thighs. Circular excisions were then made on the marked area of the skin surface and the skin carefully dissected out. The area was measured immediately by

tracing out the wound area using a transparent tracing paper and the squares counted. The animals were divided into seven groups of five animals each and were treated as follows; Batch A and B animals were treated topically with PEG400 alone and *V. amygdalina* alone respectively, whilst batches C, D, E were treated with (1:1, 1:3 and 3:1) respectively. The controls were similarly treated, F (positive control) received neomycin and G a received distilled water (negative control). The solid dispersion was topically applied once a day, starting from the day of the operation, till complete epithelization. Wound closure and epithelization time were the key parameters study in this work. The wounds were traced on every three- three days for six times and thereafter monitor until healing was complete. The percentage of wound closure was calculated. The period of epithelization was calculated as the number of days required for falling of the dead tissue completely.

Incision wound model

Rats were anesthetized and two paravertebral long incisions made through the skin of about 5.0 cm from the midline on each side of the back using sterile scalpel surgical blade size-20. The animals were treated as discussed in the excision study. The open skins were carefully stitched with a black silk-0 on alternate positions to ensure a good closure with minimal fluid loss. Batch A and B animals were treated topically with PEG-400 alone and *V. amygdalina* alone respectively, whilst batches C, D, E were treated with (1:1, 1:3 and 3:1) respectively. The controls were similarly treated, F (positive control) received neomycin and G a received distilled water (negative control) once a daily, the sutures were removed at the 8^{th} day post surgery and the tensile strength of the healed skin was assessed at the 10^{th} day as described in the previous method.[21]

Acute Toxicity Testing

In vivo toxicity of solid dispersions was studied in normal mice. Twenty four mice of either sex with average weight of 20g were randomly divided into three groups containing six mice each. Based on the wound healing results, batch C, D and E. The mice were orally administered with various preparations at dose of 20, 25 and 30 mg/kg. A control group F was added and treated with normal saline. After the administration, mice that received 30 mg of (**preparation d)** show a loss in body weight, all other groups were physically stable. The animals in this category were selected for further study. Animals were sacrificed and blood was collected, and used for haematological and liver investigations.

Haematological parameters.

The following blood parameters were investigated red blood corpuscles (RBC), white blood corpuscles (WBC), platelets and red cell indices viz., packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). Briefly, 0.5 ml of the blood was added inti 1.5 ml cuvette and was placed in the sample pot of the machine and analysed using an automatic haematological analyzer (Abacus junior, Germany).

Effect on the liver enzymes

A 1.5 ml of the sample collected in a plain bottle or serum extractor was used for the study. The sample was allowed to stand in an undisturbed bench for 1 h away from sunlight, this was followed by spinning for 5 min, the serum was separated from the clotted red cells, the resulting supernatant used for the assessment of liver integrity. Using a 3.2 microlitre of automated pipette a sample was drop on the sample spot of each LFT parameter strips (Total bilirubin, GOPT, Asparte and alanin aminotransferasese strip) and analysed using Reflotron-Plus machine (Model:SN747461).

Statistical Analysis

The resulting data were analysed statistically using the One-way Analysis of Variance (ANOVA), and the significant means were separated using the Duncan multiple range test. The probability level was 5 %.

RESULTS AND DISCUSSIONS

RESULTS

Table 1: Effect of topical application of the preparations on excision wound model

Batches	% Wound healing after surgery (in days)								Epithelization days
	0	3rd	6th	9th	12th	15th	18th	20th	
a- PEG	100	15.1±02	26.1±02	34.7±02	42.9±02	56.2±10	63.4±12	64.0±7	25
b-V.A	100	16.4±1	33.6±32	49.0±01	58.4±3	67.1±21	74.1±03	79.0±3	24
c- 1:1(P:V)	100	31.7±11	49.1±02	58.1±1	69.1±9	75.3±04	81.1±42	83.0±6	21
d- 1:3(P:V)	100	39.4±12	57.0±2	66.1±21	74.3±04	81.1±02	89.1±09	--	18±2*
e- 3:1 (P:V)	100	34.1±11	49.7±22	51.6±04	63.2±12	71.1±4	78.1±0	81.7±1	20
f- Neomycin	100	42.7±01	59.3±11	74.3±09	88.1±00	91.2±11	96.1±01	--	18±2*
g- Untreated	100	12.16±2	23.1±12	33.1±2	41.0±1	46.7±6	52.7±01	56.3±1	27**

P<0.01 as compared with control group. **Note: *P= PEG, V= V. amygdalina***

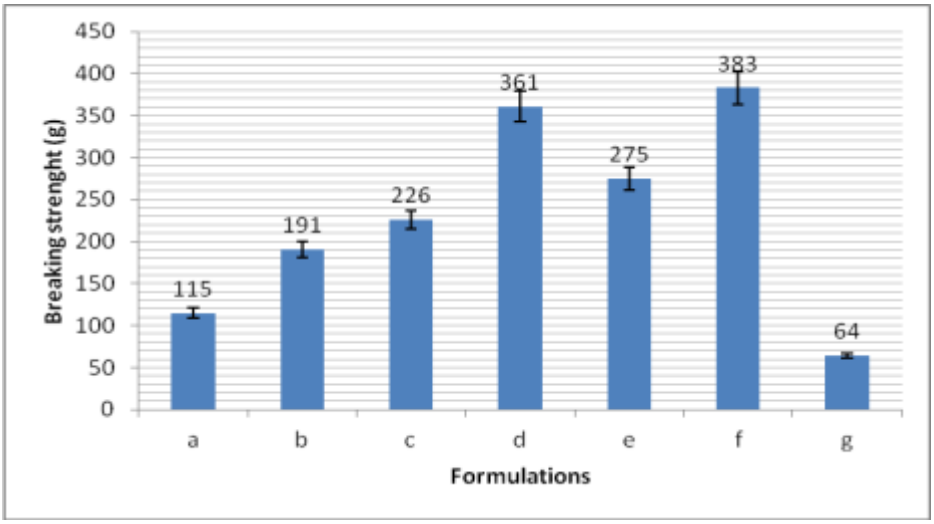

Fig. 1: Breaking strength of the excision wound model

Key: **a**= PEG, **b**=V. amygdalina, **c**= 1:1(PEG:V.A), **d**= 1:3(PEG:V.A), **e**= 3:1(PEG:V.A) **f** = Neomycin (positive control) and

g= (untreated, negative control)

Table 2. Effects of the batch on the liver enzymes and Bilirubin

Parameters	Batch E	Control
Alanine aminotransferase	17.1±2.1	15.2±0.2
Aspartate aminotransferase	12.4±1.34	9.1±2.1
Alkaline phosphatase	78.2±1.2	68.2±1.0
Bilirubin	0.95 ±1	0.71.1 ±09

P<0.01 as compared with control group

The effect of solid dispersion of PEG-4000 and methanolic extract *V. amygdalina* on incision and excision wound model, the wound healing activity of the formulation was much more than that of control (untreated). The groups treated with PEG: *V. amygdalina* in ratio (1:3) showed significant wound healing from 9th day onwards, and the rate of wound healing was comparable to that of group F treated with neomycin standard drug. The wound closer time was lesser compare to the standard drug, as well as the percentage of wound contraction of the wound. Formulation (E) with PEG:VA of 3:1 show a close activity to D, but with a variation on the wound contraction as well as the epithelization (20 days) whilst that of D is 18 days. In all the formulation and the individual component when used alone showed a better activity than the control **(Table 1)**. When *V. amygadalina* extract was compare to PEG, it was observed that the healing rate in the extract groups was more in the *V. amygdalina* than the extract, the epithelization days also follow a similar trend. The result of tensile strength

wound model is shown in **(Figure 1)**. The tensile strength group treated showed a lesser but significant increase in tensile strength compared to standard drug (positive control). The solid dispersions of the combination showed a better strength than the individual component. The results show no significant increase in the bilirubin levels between the test group and the control. The results of the enzyme study and bilirubin **(Table 2)** show no much variation as compared to the control. The activities of both alanine aminotransferase and alkaline phosphatase in the presence of the preparation showed a slightly variation compared with the control but none of these observed slight increases was statistically significant ($P>0.01$) when compared to the control. However, aspartate aminotransferase activity was significantly ($P<0.05$) when compared with the control group. There were no differences in all the heamatological parameters investigated.

DISCUSSION

Wound healing is a process consists of integrated cellular and biochemical events leading to re establishment of structural and functional integrity with regain of strength of injured tissue. Though healing process takes place by itself and need not require much help, but various risk factors such as discomfort, inability to heal by primary intention due to infection and delay in healing has brought attention to promote this process. Topical application of the solid dispersion of *V. amygdalina*-PEG on the wound site produced significant wound healing activity and increased rate of wound contraction **(Table - 1).** The healing rate noticed in (**batch d)** of the formulation is evident that the materials used in this formulation possess significant wound healing promoting activity, when compare to the individual components, but less than the positive control. It is evident that there was a synergistic effect between the PEG and the *V. amygdalina* extract when combined.

Polyethylene glycol (PEG) 4000 is a macromolecule which has been added to honey and pollen extract for the treatment of test lesions in the cow.[22] The proposed mode of action of the macromolecule is by promotion of non-adherent healing through polymer coating or siliconization of the injured surface.[23] Studies have also shown that PEG-based polymers have been extensively used for mucoadhesive applications because they exhibit high adhesive bond strengths in contact with tissues.[24-25] This increases residence time and drug bioavailability in most cases. Researchers believe that, PEG in formulation or in drug delivery systems act as mucoadhesive anchors, causing an increase in mucoadhesion and making the incorporated drug available at the delivery site.[26] Methanolic extract of *V.*

amygdalina revealed the presence of flavonoids, saponins, tannins, glycosides, sesquiterpenes and triterpenoids. These components have played various roles in the physiological process of wound healings i.e flavonoids are known to reduce lipid peroxidation and improve vascularity. It was a known fact, that lipid peroxidation increase the viability of collagen fibrils by increasing the strength of collagen fibres, through proper blood circulation and protect cell damage. Other components such as tannins, sesquiterpenes and flavonoids also assist in the process of wound healing.[27-30] The antibacterial activity of *V. amygdalina* against some gram-negative and gram-positive bacteria and it antioxidant property have been reported.[31-32] It has been suggested that bitter leaf could be effectively used against drug resistant microorganisms.[33] The negative effect cause by the bacteria on wound healing cannot be ignoring in wound healing rate. Hence, promotion of wound-healing process may also be due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelization. Thus, wound-healing activity of *V. amygdalina* may be attributed to it constituents present in it, which may be either due to their individual or additive effect that fastens the process of wound healing.[34] There was no obvious side effect on the heamatological and the liver enzymes investigated within the scope of this research. The combination of this extract and PEG-4000 a known bioadhesive biodegradable polymer as well as a drug penetration enhancer maybe the sole gain of this formulation. Hence, it good wound healing activity.

CONCLUSION

Based on the results of this present investigation, we concluded that solid dispersions of this formulation is a better way of presenting the *V.amygdalina*-PEG pharmaceutically for patients acceptance rather than traditional method of applyinmg the extract in wound healing.

DELCARATION

We declared that, the research was self sponsor and that there was no conflict of interest.

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