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HISTOLOGICAL EXAMINATION OF WOUNDS TREATED WITH CRUDE LATEX OF *Calotropis procera* STEM

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Abstract: **Evaluation of wound healing activity of crude latex of *Calotropis procera* stem was investigated in this present study. Thirty (30) rats of both sexes were divided into six groups of five animals each, Group A (Control) was administered feeds and distilled water only; group B was treated with 0% *Calotropis procera* crude latex + 100% ointment ; group C was treated with 25% *Calotropis procera* crude latex + 75% ointment; group D was treated with 50% *Calotropis procera* crude latex + 50% ointment; group E was treated with 75% *Calotropis procera* crude latex + 25% ointment and group F was treated with 100% *Calotropis procera* crude latex + 0% ointment. The results of histological examination showed that the wounds after 7 days treatment with crude *latex* of *Calotropis procera* stem exhibited marked dryness of wound edges with regeneration of healing tissue and the wound area was also considerably reduced. A near test of granulation tissue sections displayed faster tissue regeneration in the treated group compared to untreated group administered distilled water only. Also, there was marked infiltration of inflammatory cells, increased blood vessel formation and enhanced proliferation of fibroblasts in latex of *Calotropis procera* stem treated groups mostly group F which performed favourably and higher than the conventional ointment. The fresh crude latex of *Calotropis procera* stem showed practical wound healing activity which was attributed to its phytochemical constituents. The mechanism of action of wound healing property of latex of *Calotropis procera* and future research on internal wound healing like ulcer are recommended for further study.**

Keywords: **latex, ointment, wound, *Calotropis procera*, stem.**

1. INTRODUCTION

Calotropis procera, is an Ayurvedic plant with important medicinal properties. It is known by various vernacular names like Swallow wort in English, madar in Hindi, Alarka in Sanskrit, Bomu-Bomu in Yoruba and Tumfafiya in Hausa, it is found in most parts of the world with a warm climate in dry, sandy and alkaline soils (Shoaib *et al.*, 2013). Common names is, English (Apple of Sodom, rooster tree, Auricula tree, Cabbage tree, *Calotrope*, *Calotropis*, Dead Sea apple, Giant milkweed, Indian milkweed, Kapok tree, King Edward's crown, King's crown, King's crown kapok, Prince of Wales' crown, Rubber bush, Rubber plant, Rubber tree, Small crown flower, Sodom apple, Sodom's milkweed, Swallowwort); (Kumar and Arya, 2006). French (pomme de Sodome, algodón de seda,arbre á soie, cotton soie, arbre a soie du Senegal); Bomu-Bomu in Yoruba and Tumfafiya in Hausa, *(*Orwa et al. 2009).

Plant possesses gray green sessile leaves of 15-30 cm long and 2.5-10 cm broad having succulent and waxy appearance. The flowers are non-scented, small, cream or greenish white at the base and purple violet at the extremity of the lobes and pentamerous. Follicles are recurved with 2 or 1 follicles, second more often suppressed, 3-4 long (Orwa *et al.*, 2011). Calotropis is primarily harvested because of its distinctive medicinal properties. The inner bark of *Calotropis procera plant* is used to make strong fibers called madar which are used in the manufacture of weave carpets, ropes, sewing thread and fishing nets. *Calotropis procera* is an erect, tall, large, highly branched and perennial shrub or small tree that grows to a height of 5.4 m, with milky latex throughout (Shoaib *et al.*, 2013).

The chloroform extract of the root has shown to exhibit protective activity against carbon tetrachloride induced liver damage. Protein fraction of *Calotropis procera* latex protects against 5-fluorouracil induced oral mucositis associated with down regulation of pivotal pro inflammatory mediators and activates murine macrophages (Seddek *et al.*, 2010). *Calotropis procera* extract showed antioxidant and protective effects against alloxan induced diabetics and carbon tetrachloride induced liver and gastric injuries in rats (Bharti *et al.*, 2010).

Calotropis procera and *Calotropis gigantea* roots showed pregnancy interceptive and anti-fertility activity in female rats. These also showed histomorphometric and histopathological effects in male reproductive organs of Wistar rats (Akinloye *et al.*, 2002). The milky white latex obtained from the plant exhibits potent anti-inflammatory activity in various animal models that is comparable to standard anti-inflammatory drugs (Sangraula *et al.*, 2002). It shows protective effect against inflammatory hyperalgesia and reduces oxidative stress in Freund's complete adjuvant induced mono arthritis in rats (Kumar and Roy, 2007). *C. procera* ethanololic extract of the flowers is reported to have anti-inflammatory activity while latex administration in animal models induce peritonitis, paw edema, hemorrhagic cystitis, immunological and allergenic responses which are controlled by administration of differentanti inflammatory drugs (Arya and Kumar, 2004).

It also displayed much higher anti-metastatic activity against different cancerous cells and cell lines than any conventional drug and can be used for interventional therapies used in complementary and alternative medicine to cure different types of cancers (Yates *et al.*, 2005). Plant is a good source of various future herbal drugs and drug templates which might be non-steroidal and anti-inflammatory in nature and show wider cancer suppressing efficacy in cancer patients (Ali *et al.*, 2001).

The latex of *Calotropis procera* and *Calotropis gigantea* latex display a good wound healing efficacy of dermal wounds in guinea pigsn (Juncker *et al.*, 2009). Similarly, root bark of *Calotropis gigantea* shows wound-healing activity in rats (Perumal and Show, 2012). However, reports on wound healing activity of latex obtained from *Calotropis procera* stem are unavailable. It is therefore the interest of the present study to determine the histological examination of wounds treated with latex obtained from *Calotropis procera* stem

2. MATERIALS AND METHODS

MATERIALS

Collection of Experimental Animals

40 healthy Wistar albino rats (4 weeks old) of either sex with mean weight of 90.0±3.1g were obtained from the animal house of University of Nigeria Nsukka, Enugu State. The rats were divided into six cages of five rats same sex per cage for the wounding experiment and kept in the animal house of Biochemistry & Molecular Biology Department, Nasarawa State University, Keffi - Nigeria; the animals were fed *ad libitum*, allowed access to free food (Vital feed - growers mash) and water. They were acclimatized for 2 weeks in the new environment.

Collection of Plant sample

The collection of fresh crude latex was done by making small incisions on stem of matured *Calotropis procera* plant plant within Nasarawa State University, Keffi, Nigeria, allowing the crude latex to flow into sterile plastic bottles. The crude latex was gently handled to maintain its integrity during transport to the laboratory. The crude latex was then refrigerated at 25^{o}C until needed.

METHODS

Determination of Quantitative and Qualitative Phytochemical Content

The procedures decribed by Okerulu *et al.*, (2017) and Sofowora (1989) were used for the qualitative and quantitative determination of phytochemical contents of the latex of *Calotropis procera* stem.

Test for Tannins

About 0.1 g of crude latex sample was boiled in 4ml of water in a test tube and then filtered. Few drops of 0.1% ferric chloride were added to observe brownish green or blue-black coloration indicative of the presence of tannins.

Saponins

1ml of crude latex of *Calotropis procera* was poured in test tubes; shook dynamically to form a stable froth, followed by addition of six drops of olive oil to this sample. Formation of an emulsion revealed the presence of saponins.

Test for Flavonoids

About 3ml of dilute ammonia was added to 2ml of crude latex of *Calotropis procera*. This was followed by addition of 1ml concentrated Sulphuric acid (H_2SO_4). Yellow coloration in each extract showed the presence of flavonoids.

Steroids

10ml chloroform was added in 1ml of each extract in a test tube. After that 10ml concentrated sulphuric acid was dissolved in this test tube. Two layers were formed; lower layer expressed yellow color along green fluorescence while upper layer showed red. The formation of these layers indicates steroids were present.

Alkaloids

To 2ml of each fraction, 2ml of conc. HCl was added and then few drops of Mayer's reagent were mixed to it. Formation of white precipitate or green color indicated the presence of alkaloids.

Phenols

To the 1ml of crude latex of *Calotropis procera*, 2ml of distilled water and three drops 10 % $FeCl_3$ were added. Formation of blue green color was showing phenol presence.

Grouping of Experimental Animals

According to (Jones *et al.*, 2004) animals were divided into 6 cage groups of 5 rats each for the wounding experiment.

Group A: This group was administered distilled water only

Group B: This group was treated with 0% *C. procera* crude latex + 100% Penicillin (0:4) mL

Group C: This group was treated with 25% *C. procera* latex + 75% Penicillin (1:3) mL

Group D: This group was treated with 50% *C. procera* latex + 50% Penicillin (1:1) mL

Group E: This group was treated with 75% *C. procera* latex + 25% Penicillin (3:1) mL

Group F: This group was treated with 100% *C.procera* latex + 0% Penicillin (4:0) mL

Determination of Excision Wounds Model

According to Narendra *et al.*, (2009), the animals were anesthetized with slight vapor inhalation of diethyl ether and the right side of each rat was shaved and disinfected with 70% alcohol. Excision wounds sized 300 mm^2 and 2 mm depth was made by cutting out layer of skin from the shaven area using sterile surgical blade. The entire wound was left opened. The treatment was done topically in all the cases. The treatment lasted for 16 days of various concentrations. Wound areas was measured on days 1, 5, 9, 13 and 16 for all groups, using a transparency paper and a permanent marker over the wound and tracing it out. The parameter studied was percentage wound contraction calculated using the following formula: percent wound contraction = (original wound area -unhealed area)/original wound area ×100

HISTOLOGICAL EXAMINATION

The experiment was terminated after 16th day and the wound was removed surgically for histopathological examination. The tissues were cut and trimmed down and processed in dehydration in alcohol series 50–90% for 30min each, 100% ethyl alcohol (I/II), and histoclear 100% (I) for 60 min each, and 100% histoclear (II) for overnight (Ramar and Vincent, 2012). The decalcification was done with molted wax at 55◦C for 1 h in each jar. The blocks were prepared by using wax, 5 μm thick sections were stained with haematoxylin and eosin (H&E) and imaged by Olympus Light microscope (Ramar and Vincent, 2012).

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Tissue preparation

Sections of the liver, kidney and skin were collected for histopathological examination. The samples were fixed in preservative for a minimum of 48 hours. The tissues were subsequently trimmed, dehydrated in 4 grades of alcohol (70%, 80%, 90% and absolute alcohol), cleared in 3 grades of xylene and embedded in molten wax. On solidifying, the blocks were sectioned, 5µm thick with a rotary microtome, floated in water bathe and incubated at 60˚C for 30 minutes. The 5µm thick sectioned tissues were subsequently cleared in 3 grades of xylene and rehydrated in 3 grades of alcohol (90%, 80% and 70%). The sections were then stained with Hematoxylin for 15 minutes (Ramar and Vincent, 2012). Blueing was done with ammonium chloride. Differentiation was done with 1% acid alcohol before counterstaining with Eosin. Permanent mounts were made on degreased glass slides using a mountant; DPX (Ramar and Vincent, 2012).

Slide Examination

The prepared slides were examined with a Motic™ compound light microscope using x4, x10 and x40 objective lenses (Ramar and Vincent, 2012). The photomicrographs were randomely taken using a Motic™ 5.0 megapixels microscope camera at x160 magnifications. SKIN (Photomicrograph captured at magnifications x100; x160)

STATISTICAL ANALYSIS

Data obtained were presented as Mean ± SEM of mean was value of the determinations, one-way analysis of variance (ANOVA) was used to test for significance at 5% confidence level.

3. RESULTS AND DISCUSSION

Quantitative and qualitative phytochemical analysis of latex of *Calotropis procera* stem.

The results of quantitative and qualitative phytochemical analysis of crude latex of *Calotropis procera* stem are presented in Table 1. The results showed mean values of 912.09+4.41 mg/100g, 0.33+0.06 mg/100g, 1170.16+98.22 mg/100g, 0.86+0.03 mg/100g, 610.71+10.48 mg/100g, and 707.50+108.78 mg/100g were recorded for tannins, saponins, flavonoids, steroids, alkaloids and phenols respectively. Similarly, tannins, flavonoids, alkaloids and phenosl were excess while saponins and steroids were trace in the latex of *Calotropis procera* stem. The highest concentration of the phytochemical in the crude latex of *Calotropis procera* stem was flavonoids while the least phytochemical content was saponins.

Excision wounds model (Percentage wound closure/contraction)

The results of percentage wound closure on days 5, 9, 13 and 16 day are presented in Table 2. The result of day 5 showed that the percentage mean values of 6.84 ± 1.11%, 10.86 ± 1.64%, 14.96 ± 1.48%, 16.53 ± 2.60%, 13.00 ± 4.50% and 13.03 ± 3.54% were recorded for groups A, B, C, D, E and F respectively. On day 9, percentage mean values of 14.29 ± 4.19%, 32.63 ± 8.40%, 31.23 ± 8.97%, 35.54 ± 8.51%, 42.19 ± 5.10% and 46.79 ± 2.16% were recorded for groups A, B, C, D, E and F respectively. Also, that of day 13, percentage mean values of 35.61 ± 4.25%, 68.25 ± 6.31%, 73.46 ± 3.16%, 77.39 ± 3.03%, 72.55 ± 5.72% and 89.02 ± 3.77% were recorded for groups A, B, C, D, E and F respectively. Similarly, that of day 16, percentage mean values of 45.63 ± 4.48%, 84.98 ± 5.95%, 89.53 ± 5.95%, 89.58 ± 1.79%, 91.61 ± 2.79% and 100.00 ± 2.27% were recorded for groups A, B, C, D, E and F respectively.

Histopathological Examination

Normal histology

Sections of the skin presented in this group showed the normal histology of the skin. Normal epidermis (E), dermis (D), hair follicles (Arrow) and sebaceous glands (G) were observed as shown in Figure 1.

Toxic effects on Skin plug of Wistar albino

Sections of the skin presented in this group showed a wide area of incomplete wound closure (black arrow) with marked dermal inflammatory cellular infiltration (CI). There is also incomplete involution of the granulation tissues in the deep dermis (D), persisted angiogenesis (red arrow) with hemorrhaging (yellow arrow) as shown in Figure 2.

In group A, a wide area of incomplete wound closure covered by thick scab (black arrow) as well as moderate to marked dermal inflammatory cellular infiltration (CI) was observed. There was incomplete involution of the granulation tissues in the deep dermis (D), persisted angiogenesis (red arrow), Dermis (D) as shown in Figure 3.

In group B, there were wide area of incomplete wound closure covered by thick scab (black arrow) with moderate to marked dermal inflammatory cellular infiltration (CI) and an incomplete involution of the granulation tissues in the deep dermis (D), persisted angiogenesis (red arrow), Dermis (D). As shown in Figure 4.

Sections of the skin presented in group C showed complete wound closure with re-epithelization of the epidermis (E). In the dermis (D), there was complete involution of the granulation tissue and replacement with fibrous connective tissue. Evidence of remodeling with formation of hair follicles and associated structures were observed (arrow) as shown in Figure 5.

Skin plug of animals in group D showed complete wound closure with re-epithelization of the epidermis (E). In the dermis (D), there was complete involution of granulation tissue and replacement with fibrous connective tissue. Evidence of remodeling (arrow) with formation of hair follicles and presence of chronic inflammatory cells in the dermis were observed as shown in Figure 6.

Sections of the skin presented in group E showed complete wound closure with re-epithelization of the epidermis (E). In the dermis (D), there were complete involution of the granulation tissue and replacement with fibrous connective tissue. However, dermal hemorrhages were still evident (arrow) as shown in Figure 7.

Sections of the skin presented in group F showed complete wound closure with re-epithelization of the epidermis (E). In the dermis (D), there were complete involution of the granulation tissue and replacement with fibrous connective tissue as shown in Figure 8.

Saponins are used as anti-fungi agent and also as industrial adjuvants (Adejumo & Ajiboso, 2003). Steroids were found to be low in concentration; steroids are used in the stimulation of bone marrow and growth. It stimulates lean body mass and also play vital roles in the prevention of bone loss in elderly men (Yamamato and Gaynor, 2000). Tannins have been reported as antibacterial aggents, tannins decrease bacterial proliferation. Phenols have potential for beneficial effects on health by blocking key enzymes at microbial metabolism. Flavonoids was found to be highest, reported from previous study shows that flavonoids possess anti-viral, anti-inflammatory, antioxidant activity, cytotoxic and also used in the treatment of hypertension, diabetes, rheumatic fever (Usoh *et al.*, 2005; Tolulope, 2007). Alkaloids also interfere with cell division; hence the presence of alkaloids in the plant makes it a possible remedy in the treatment of cancer (Adejumo & Ajiboso, 2003).

Wound contraction is beneficial as it can significantly reduce healing time because less granulation tissue needs to be produced to replace tissue loss (Calvin, 1988). According to Tejero-Trujeque (2001), contraction occurs when the wound edges move towards each other in a centripetal fashion thus reducing the wound's dimensions. Excision wound model showed significant increase in percentage wound closure with the treated group mostly group F, there was increase as wound edges move toward each other. Contraction involves a dynamic process where cells organize their surrounding tissue matrix to reduce normal healing time by shrinking the amount of extracellular matrix (ECM) that needs to be produced (Jones *et al.*, 2004).

In the dermis (D), there is complete involution of the granulation tissue and replacement with fibrous connective tissue. Angiogenesis improves circulation of oxygen and nutrients essential for the healing process that including re-epithelization and evidence of remodeling with formation of hair follicles and presence of chronic inflammatory cells (Szabo *et al*.,1995). Healed wound of treated group contained a large amount of fibroblast proliferation, collagen synthesis, and neo vascularization, which resulted in an increased wound tensile strength and accelerated healing wound (Al-Henhena *et al.*, 2011).

Histologically revealed the presence of infiltration of inflammatory cells in the treatment groups hence enhanced angiogenesis and fibrogenesis as compared to control wounds and even performed better than the standard ointment. The more the percentage or ratio (concentration) of crude latex of *Calotropis procera* stem the more the effectiveness in wound healing.

Collagen confers strength and integrity to the tissue matrix and also plays an important role in haemostasis and in epithelisation at later phase of wound healing (Clark, 1996; Satyanarayana and Chakrapani, 2011). Hence enhanced collagen synthesis by latex of *Calotropis procera* stem may significantly contribute to healing and also provide necessary strength to repaired tissue (Rasik *et al*, 1999). The wounds after 7 days treatment with crude *latex* of *Calotropis procera* stem

exhibited marked dryness of wound edges with regeneration of healing tissue and the wound area was also considerably reduced compared to untreated group thus indicating the healing potential of our findings, this correlate very well with the histological findings of earlier study of Rasik *et al*, (1999). A near test of granulation tissue sections displayed that the tissue regeneration was much faster in the treated group compared to untreated group administered distilled water only. There was marked infiltration of inflammatory cells, increased blood vessel formation and enhanced proliferation of fibroblasts as a result of treatment with latex of *Calotropis procera* stem.

4. CONCLUSION

The fresh crude latex of *Calotropis procera* stem showed practical wound healing activity which was attributed to its important phytochemical counstituents. Histologically, presence of infiltration of inflammatory cells in the treatment groups mostly 100% latex treated group F, was established, hence enhanced angiogenesis and fibrogenesis when compared to untreated and even performed than Penicillin treated groups.

5. RECOMMENDATIONS

The mechanism of action of wound healing property of latex of *Calotropis procera* stem should be recommended for further study. Future research on internal wound healing like ulcer is another aspect to research on.

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APPENDICES - A

Table 1: Quantitative and Qualitative phytochemical composition of crude latex of *Calotropis procera* stem

	Quantitative Concentration (mg/100g)	Qualitative
Tannins	912.10 ± 4.41^{a}	+++
Saponins	0.33 ± 0.06^{b}	+
Flavonoids	1170.16 ± 98.22^{a}	+++
Steroids	0.86 ± 0.03^{b}	+
Alkaloids	610.71 ± 10.48^{c}	+++
Phenols	707.50 ± 108.78^{d}	+++

Keys: + means trace, ++ means abundant while +++means excess

Results are presented in Mean ± SEM, (n = 3), mean values with different superscripts down the groups are significant difference ($p < 0.05$)

Table 2: Excision wounds model (% wound closure/contraction)

Groups	Day 5	Day 9	Day 13	Day 16
Group A	6.84 ± 1.11^{a}	14.29 ± 4.19^{a}	35.61 ± 4.25^{a}	45.63 ± 4.48^{a}
Group B	10.86 ± 1.64^{b}	32.63 ± 8.40b	68.25 ± 6.31^{b}	84.98 ± 5.95^{b}
Group C	14.96 ± 1.48^{c}	31.23 ± 8.97^{c}	73.46 ± 3.16^{c}	89.53 ± 5.95^{c}
Group D	16.53 ± 2.60^{d}	35.54 ± 8.51^{d}	77.39 ± 3.03^{d}	89.58 ± 1.79^{d}
Group E	13.00 ± 4.50^{e}	42.19 ± 5.10^{e}	72.55 ± 5.72^{e}	91.61 ± 2.79^{e}
Group F	13.03 ± 3.54^{f}	46.79 ± 2.16^{f}	89.02 ± 3.77^{f}	100.00 ± 2.27^{f}

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Results are presented in Mean ± SEM, (N = 3), mean values with different superscripts across the days are considered at p< 0.05. . Group A= feeds and distilled water only, group B= 0% *Calotropis procera* crude latex + 100% Penicillin (0:4) ml, group C= 25% *Calotropis procera* crude latex + 75% Penicillin (1:3) ml, group D= 50% *Calotropis procera* crude latex + 50% Penicillin (1:1) ml, group E= 75% *C. procera* crude latex + 25% Penicillin (3:1) ml, group F=100% *Calotropis procera* crude latex + 0% Penicillin (4:0) ml

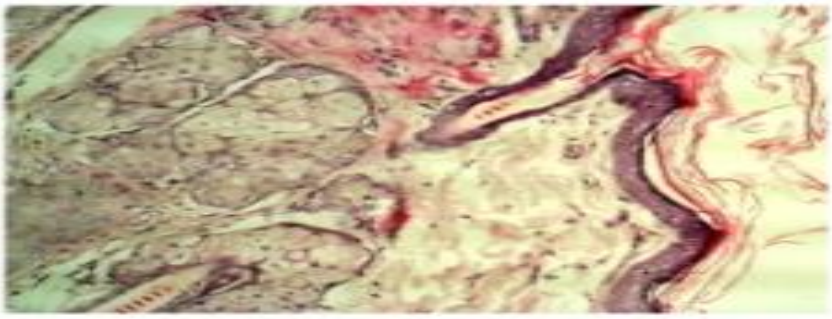

Figure 1: Normal histology

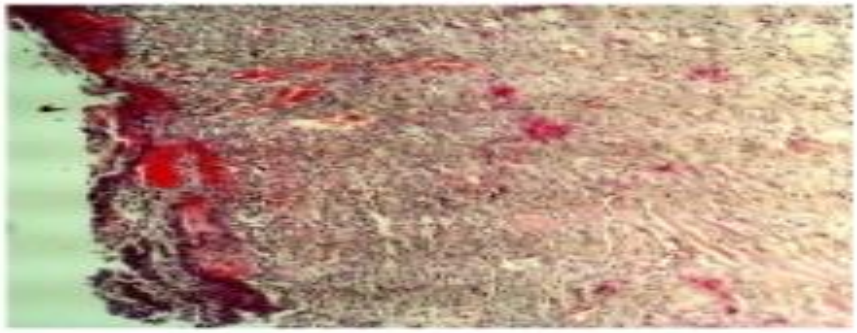

Figure 2: Toxic effects on skin plug of animal

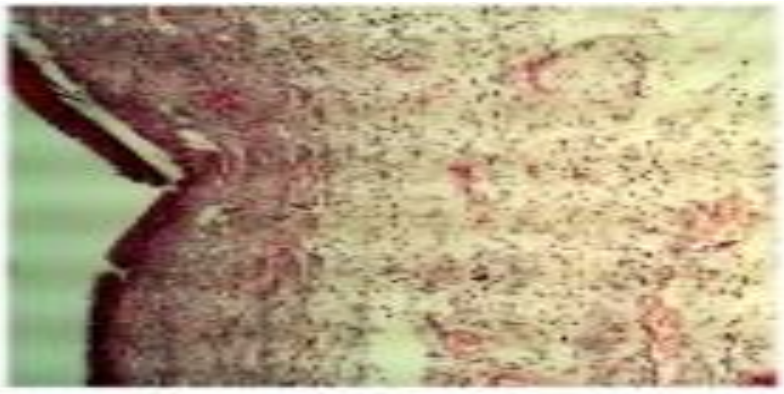

Figure 3: Group A

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Figure 4: Group B

Figure 5: Group C

Figure 6: Group D

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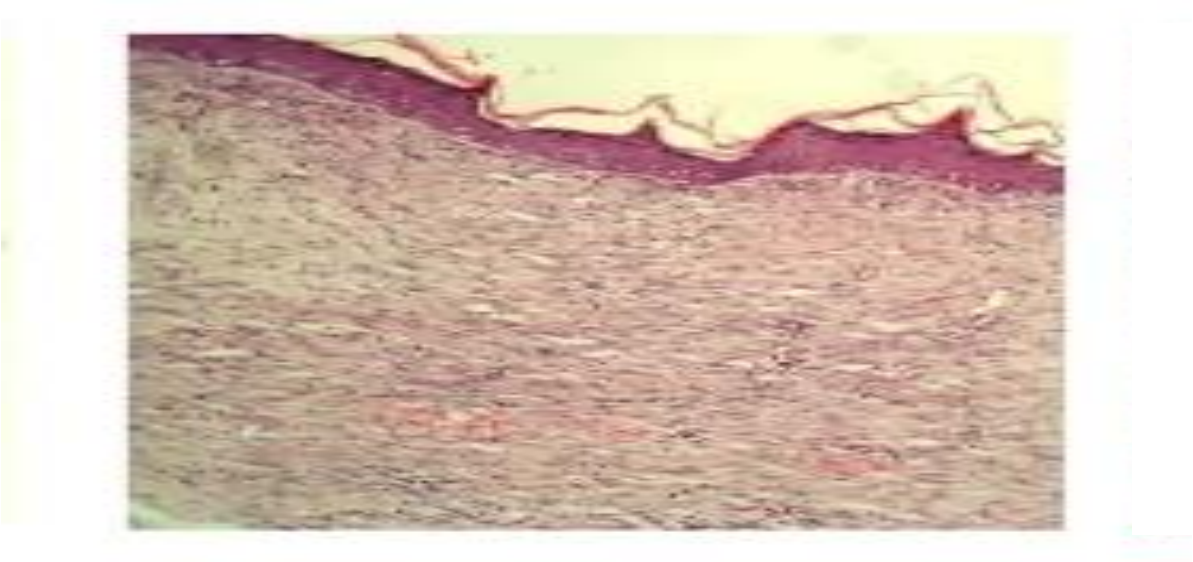

Figure 7: Group E

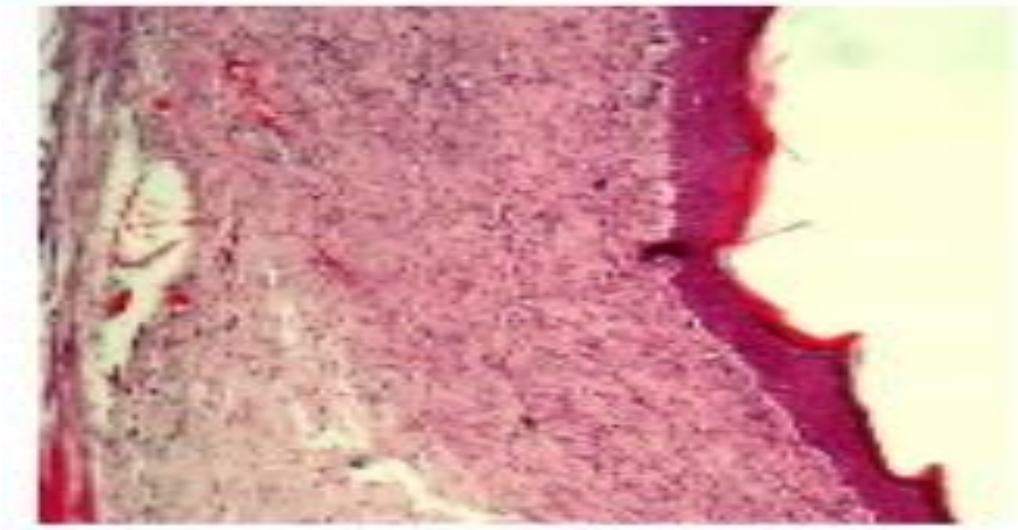

Figure 8: Group F