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Evaluation of wound healing potential of different leaf extracts of *Pupalia lappacea*

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The wound healing process is a complex and dynamic process of restoring cellular structures and tissue layers upon damage. *Pupallia lappacea* (L.) Juss is an annual or perennial herb of the family Amaranthaceae widespread in the tropics and sub-tropical regions and used in folklore medicine for treatment of boils, chronic wounds and skin infections. The study aims to determine the antioxidant and *in vivo* wound healing properties of *P. lappacea*. Extracts of leaves and aerial parts of *P. lappacea* were obtained by extracting with ethanol, petroleum ether and chloroform using the cold maceration method for 72 h. The antioxidant properties of the extracts were determined by the DPPH free radical scavenging method and the IC_{50} determined. The wound healing studies was conducted using excision wound healing model using Sprague-Dawley rats and the percentage wound closure determined for 11 days. Histological studies of wound tissues revealed appreciable collagenation, re-epithelialisation, granular tissue formation and angiogenesis for wounds treated with 2 and 10% w/w ethanol leaf extract creams as well as 1% chloroform extract creams to compared untreated wound tissues. All the extracts exhibited some level of antioxidant activity with IC_{50} values of 9.67, 17.37 and 63.27 µg/mL for the chloroform, ethanol and petroleum ether extracts, respectively. The ethanol and chloroform extracts also exhibited high rate of wound closure with 33.60% ± 2.29 to 67.23% ± 1.90 ($p \leq 0.001$) and 35.65% ± 2.30 to 67.68% ± 1.62 ($p \leq 0.001$), respectively from the 7th day post wounding to the 11th day as compared to the untreated wounds.

Key words: Excision wound model, antioxidant, wound contraction, histological studies.

INTRODUCTION

Wound healing is a dynamic and orderly progression of events and processes that establish the integrity of damaged tissues. The process basically involves three overlapping phases; inflammation, tissue formation and tissue remodeling (Singer et al., 1999; Barua et al., 2009). The minimization of tissue damage, debriding of

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non-viable tissue, maximization of tissue perfusion and oxygenation are some factors that have been recognized to enhance the wound healing process (Barua et al., 2009). *Pupalia lappacea* (L.) Juss is an annual or perennial herb of the family Amaranthaceae. The shrub is widespread in the tropics and sub-tropical regions and used in folklore medicine for the management of boils and chronic wounds (Neeharika et al., 2013). The leaf paste of *P. lappacea* with edible oil is used to treat bone fractures and inflammatory conditions (Ravi et al., 2012). The plant has been found to contain 1-docosanol, stearic acid, stigamasterol, β-sitosterol, saropeptate (N-benzoyl-L-phyenylalaninol acetate), β-sitosterol-3-0-D–glucopyranoside, stigmasterol-3-0-β-D-glucopyra-noside and 20-hydroxylecdysone (Naidu and Rajesh, 2014). The plant has been shown to possess very good antioxidant and anticancer properties (Aladedunye et al., 2002; Ravi et al., 2012). It has been found to exhibit good antinociceptive and antipyretic properties (Neeharika et al., 2013). The study aims at evaluating the wound healing potential of different extracts of *P. lappacea.*

MATERIALS AND METHODS

Collection of plant material

The leaves and aerial parts of *P. lappacea* was collected in the month of May, 2012 at Kuntunase in the Bosomtwi District of the Ashanti Region of Ghana. It was authenticated by Dr. G. H. Sam and a voucher specimen with number (KNUST/HMI/2013/L006) has been deposited at the Department of Herbal Medicine Herbarium, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Extraction of plant material

The plant sample was washed of foreign materials and dried at room temperature (25 to 30°C). The dried plant sample was milled into coarse powder in a lab mill machine (Christy and Norris, England, UK). An amount of 600 g of the powdered plant sample was taken and divided into three portions each weighing 200 g. The various portions were extracted separately with 1.0 L quantities of chloroform (BDH, England, UK), petroleum ether (BDH, England, UK) and 70% ethanol (Scharlau, England, UK) by cold maceration for 72 h amidst stirring with a glass rod at selected time intervals. After 72 h, the supernatant was extracted using a cheese cloth and the bulk extract beneath was filtered with a Whatmann filter paper (BDH, England, UK) with the aid of a vacuum pump. The extract obtained was concentrated with the aid of a rotary evaporator (Buchi, Disendorf, Germany) at 40°C and lyophilized. The weights of the extracts were recorded. The dried extracts were stored at 4°C in the refrigerator (Sharp, UK) until ready for use.

Phytochemical screening

Phytochemical screening of some secondary metabolites was conducted on the powdered plant material and the extracts for the presence of sterols, flavonoids, anthracene glycosides, saponnin glycosides tannins and alkaloids (Evans, 2009).

Determination of HPLC profile of extracts

The HPLC profiles for the extracts were determined for the purposes of identifying the extracts in addition to the phytochemical screening. The chromatograms were determined using an HPLC with UV detector at 254 nm. The chromatographic conditions included a flow rate of 1 mL/min and a pressure of 21 MPa. Extract concentration of 10 mg/mL was used for the determinations. A quantity of 10.0 µL was injected. The retention times and area under curve (AUC) produced by the various peaks (constituents) were observed and recorded as HPLC profiles of the extracts.

Determination of antioxidant activity

Free radical scavenging method

The antioxidant properties of the extracts were evaluated according to the method described by Braca et al. (2001). Extracts and α-tocopherol (Sigma-Aldrich, Steinheim, Germany) solutions of concentrations of 1.56, 3.12, 6.25, 12.5, 25.0, 50.0 and 100.0 µg/mL were prepared with methanol (BDH, England, UK). Concentration of 5.0×10^{-6} M solution of DPPH (Sigma Aldrich, Steinheim, Germany) was prepared with methanol in a dark room. 3 mL of the DPPH solution was added to 1 mL of the extracts and standard concentrations prepared and kept in the dark for 30 min. The absorbance of the DPPH in methanol (Aa) and DPPH plus the extract (Ab) was measured at 517 nm. The percentage free radical scavenging activity was calculated from the equation

$[(Aa - Ab) / Aa] \times 100\%$.

Inhibition concentration, IC_{50} was determined as the concentration which scavenged 50% of free DPPH radicals.

In vivo wound healing studies

Ethical clearance

The excision wound model studies were approved by Animal Ethical Committee, Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana and performed in accordance with the guide for care and use of laboratory animals.

Laboratory animals

The experiments were performed using 45 healthy male Sprague-Dawley rats with weights between 180 to 220 g. The rats were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The animals were kept in stainless steel cages under standard environmental conditions of temperature (30 ± 2°C) with a twelve hour cycle of light and darkness, and adequate humidity. The animals were fed with standard pellet diet (GAFCO, Tema, Ghana) and provided with water *ad libitum*.

Experimental design

The wound healing experiment was performed using the chloroform

Table 1. Phytochemical screening of dried powdered *P. lappacea* and its extracts.

Secondary metabolite	Powdered *P. lappacea*	Pet ether leaf extract (PLP)	Chloroform leaf extract (PLC)	Ethanol leaf extract (PLE)
Alkaloids	+	+	+	+
Saponin glycosides	+	-	-	+
Anthracene glycosides	-	-	-	-
Flavonoids	+	+	+	+
Sterols	+	+	+	+
Condensed tannins	+	+	+	+
Hydrolysable tannins	-	-	-	-

+ = present; - = absent.

Table 2. Free radical scavenging activities of leaf extracts of *P. lappacea*.

Extract	PLC	PLP	PLE	α- tocopherol
IC_{50} (µg/mL)	9.67	63.27	17.37	0.155

(PLC) and ethanol (PLE) leaf extracts of *P. lappacea*. The rats were divided into nine groups each consisting of five rats: group I: untreated (negative control), group II: treated with aqueous cream base (vehicle), group III: treated with 1% w/w silver sulphadiazine (Aryton Drugs, Accra, Ghana) (Agyare et al., 2014), groups IV, V and VI were treated with 0.25, 0.5 and 1.0% w/w PLC creams, respectively. Groups VII, VIII and IX were treated with 2.5, 5.0 and 10.0% w/w PLE creams, respectively. The creams were applied topically onto the wound surfaces daily after cleansing with normal saline solution for 11 days. Wound scar tissues were cut on the 12th day post wounding for histopathology studies.

Excision wound healing model

In vivo wound healing studies was performed according to the excision method described by Morton and Malone (1972). The dorsal fur of the rats was shaved using razor blades; the shaved area was then cleaned with 70% ethanol (Scharlau, England, UK). The rats were anaesthetized with an intramuscular dose of 50 mg/kg body weight of ketamine hydrochloride (Pfizer Pharmaceuticals, New York, USA). With a pair of sterile scissors excision wounds of approximately 20 mm in diameter were made at the back of each rat. The wounds were left untreated for a period of 24 h. The extracts and reference drugs were applied topically at the wound site. The diameter of the excised wounds were monitored every 48 h and measured until the 12th day.

Histological investigations

Wound tissues were cut on the 12th day post wounding from all the groups and fixed in Bouin's solution. The fixed tissues were dehydrated through increasing grades of ethanol and embedded in paraffin wax. The tissues were then cut to 5 µm sections with a rotary microtome, deparaffinised, mounted on clean glass slides and stained with haematoxylin and eosin. The glass slides were then observed under the microscope for histopathology changes. The sections were observed for the degree of cell repair; granulation tissue replacement, re-epithelisation, angiogenesis and collagen formation (Chung, 1998; Udupa et al., 1995; Talekar et al., 2012).

Statistical analysis

All results were plotted and analyzed with GraphPad Prism 5.0 for windows (GraphPad Software, San Diego, CA, USA). Data was presented as mean ± standard deviation (SD) and analyzed by two-way ANOVA followed by Bonferroni post-test analysis which recognises $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as statistically significant.

RESULTS

Phytochemical screening and HPLC profiles of extracts

Phytochemical screening revealed the presence of alkaloids, flavonoids, sterols and condensed tannins in the dried powdered leaf material of *P. lappacea* and its chloroform and ethanol leaf extracts (Table 1). The HPLC profiles of the extracts are recorded (Figure 1).

Antioxidant activity

All the extracts (chloroform, ethanol and petroleum ether) exhibited some level of antioxidant properties with IC_{50} values of 9.67, 17.37 and 63.27 µg/mL, respectively (Figure 2 and Table 2).

Table 3. Percentage wound closure of chloroform leaf extract of *P. lappacea.* PLC= chloroform leaf extract of *P. lappacea*.

Time/day	PLC 1	PLC 0.5	PLC 0.25	Vehicle	1% S/S	Untreated
1	5.494±1.396	2.788±1.515	3.904±1.859	1.621±0.504	2.93±0.695	2.82±0.282
3	8.157±2.319	8.973±3.051	10.994±4.264	1.368±1.021	4.96±1.855	2.758±0.935
5	18.824±1.317	18.636±1.484	18.964±4.042	7.422±1.124	16.07±1.847	10.35±0.965
7	38.815±2.306	44.114±2.629	35.651±5.427	27.154±4.777	32.096±2.747	22.123±0.844
9	57.813±1.381	61.744±1.849	46.810±8.580	42.616±2.597	52.362±3.092	41.783±1.606
11	66.200±2.219	67.679±1.623	59.294±5.039	64.224±5.143	65.936±3.551	50.039±1.896

PLC= chloroform leaf extract of *P. lappacea*; PLC 1=1% w/w cream; PLC 0.5= 0.5% w/w cream; PLC, 0.25% w/w cream; S/S=silver sulphadiazine; N=5 rats per group; *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant compared with the untreated wounds.

Table 4. Percentage wound closure of ethanol leaf extract of *P. lappacea*. PLE= ethanol leaf extract of *P. lappacea*; PLE 10= 10% w/w cream; PLE 5= 5% w/w cream; PLE 2= 2% w/w cream; S/S= 1 % w/w silver sulphadiazine; N=5 rats per group.

Time/day	PLE 10	PLE 5	PLE 2	Vehicle	1%S/S	Untreated
1	2.817±1.462	2.113±1.331	2.416±0.709	1.621±0.504	2.93±0.695	0.282±0.282
3	9.042±1.758	5.696±2.481	4.916±1.819	1.368±1.021	4.96±1.855	2.758±0.935
5	16.911±1.653	16.909±3.443	15.934±3.461	7.422±1.124	16.07±1.847	10.35±0.965
7	33.594±2.292	29.672±2.268	38.706±5.126	27.154±4.777	32.096±2.747	22.123±0.844
9	54.913±.596	52.163±3.317	50.062±3.449	42.616±2.597	52.362±3.092	41.783±1.606
11	65.394±2.520	60.307±2.970	67.127±1.900	64.224±5.143	65.936±3.551	50.039±1.896

Wound closure

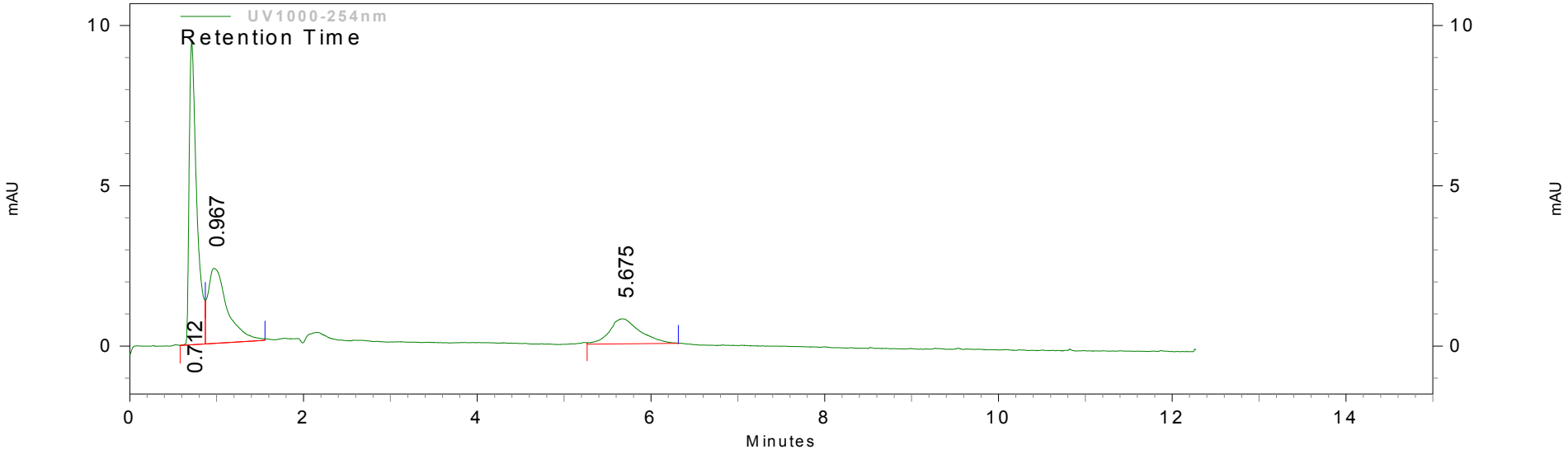
All concentrations of the extracts (0.25, 0.5, 1.0% w/w PLC and 2, 5, 10% w/w PLE) demonstrated wound healing effects compared to the untreated and the vehicle. Two and 10% w/w PLE demonstrated significant wound healing activities ($p < 0.001$ and $p < 0.05$) on days 7, 9 and and 11. 1 and 0.5% w/w PLC also demonstrated significant ($p < 0.001$ and $p < 0.05$) wound healing activities on days 7, 9 and 11 as compared to the untreated and vehicle only (Figures 3 and 4, Tables 3 and 4).

Histological studies

The histopathology evaluation of the wound tissues revealed a high level of collagen formation, tissue granulation and re-epithelialization for wounds treated with 2.0, 10% w/w PLE and 0.5% w/w PLC as compared to the untreated wounds and the wound treated with the vehicle. Wounds treated with 5% w/w PLE had high oedematous tissue with diffused pus evident of persistent tissue necrosis. Wounds treated with 0.25% w/w PLC exhibited delayed wound healing with increased fibrosis and profuse angiogenesis and vascularity. The untreated wound demonstrated persistent inflammation with marked tissue necrosis. The wound treated with the cream base only also exhibited persistent inflammation with evidence of chronic inflammation but with appreciable re-epithelialization (Figure 5).

DISCUSSION

Medicinal plants have been used for decades as cure for various human diseases because they contain various compound of therapeutic value. The study on *P. lappacea* revealed some biological activities of the plant. The antioxidant potential of the extracts of *P. lappacea* demonstrated some level of activity. Oxygen plays an important role in the healing of wounds. Excess as well as lack of oxygen can be very detrimental to the healing of wounds (Guo and DiPietro, 2010). Over-production of reactive oxygen species (ROS) at wound sites results in the oxidative stress which results in impaired wound healing. Prolongation of this process leads to tissue necrosis. Removal of these reactive oxygen species from wound sites will therefore enhance the wound healing process (Shetty et al., 2008). Antioxidants play a vital role in the elimination of these reactive oxygen species and their presence in wound healing agents is important to enhance the healing of wounds. The results from the screening revealed that the extracts of *P. lappacea* demonstrated antioxidant properties. The IC_{50} values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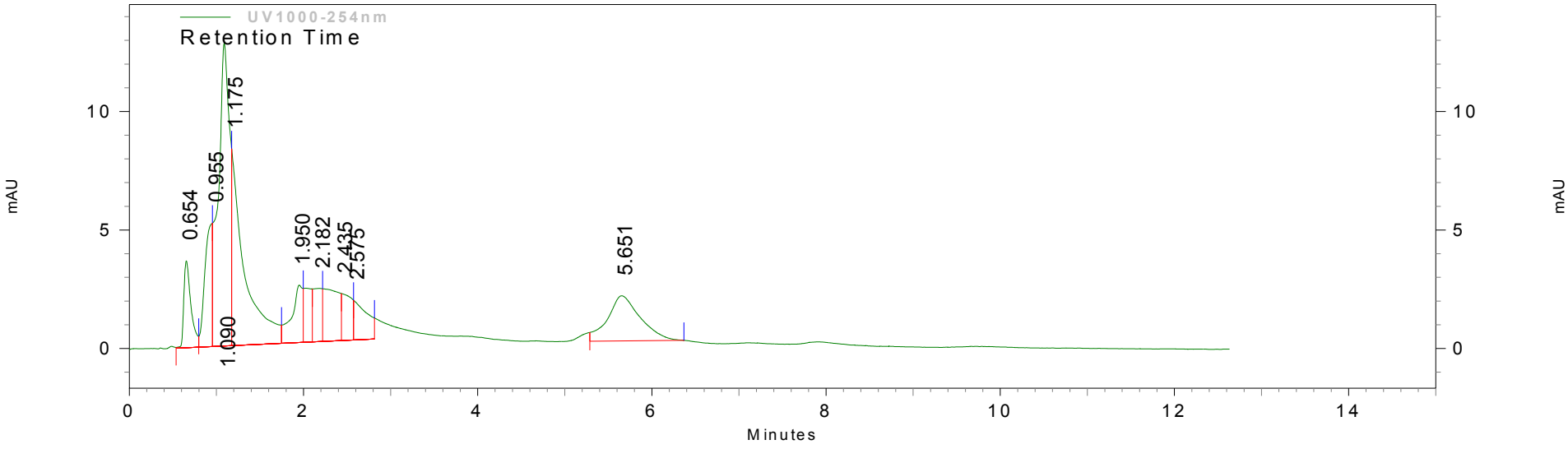
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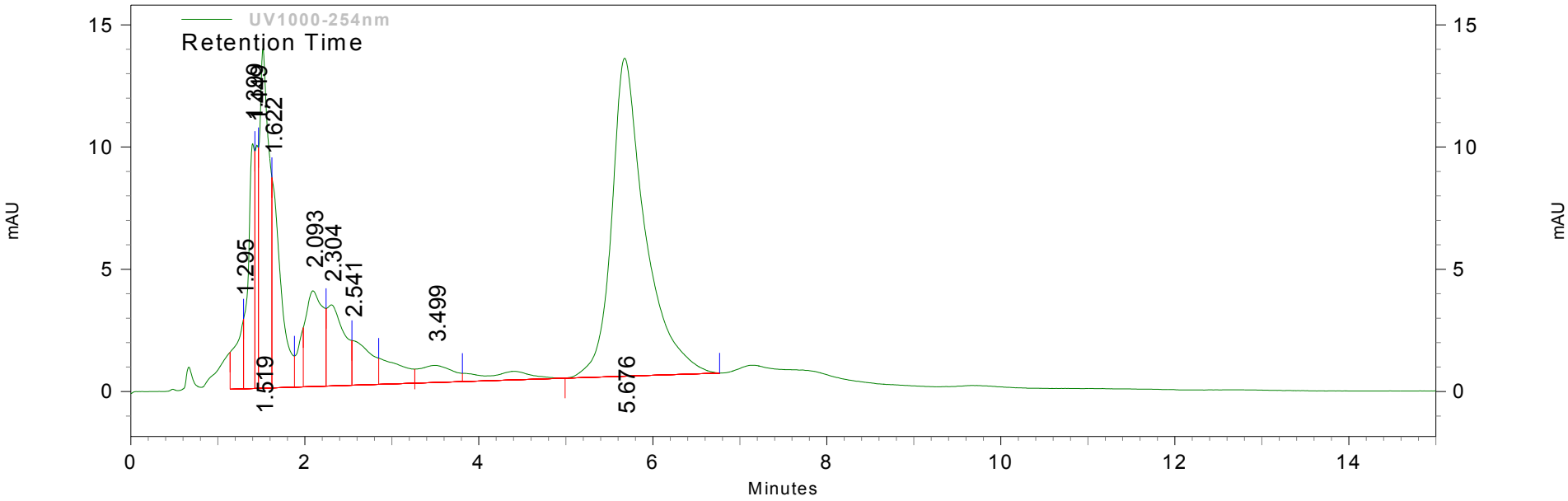

Figure 1. HPLC profiles of pet ether leaf extract (**A**), chloroform leaf (**B**) and ethanol leaf (**C**) extracts of *P. lappacea.* IC_{50} – concentration that gives 50% reduction in DPPH; PLC = chloroform leaf extract of *P. lappacea*; PLP = pet ether leaf extract of *P. lappacea*; PLE = ethanol leaf extract of *P. lappacea.*

determined for PLC, PLE, PLP and α-tocopherol indicate that α-tocopherol had the highest antioxidant property since a minimal concentration of 0.156 µg/mL will be required to scavenge 50% of free radicals (Table 2 and Figure 2). This justifies its use as the standard antioxidant.

With reference to the extracts, PLC exhibited the highest antioxidant activity since its IC_{50} values were lower than that of PLE and PLP. This could imply that majority of the phytochemicals responsible for the antioxidant properties are more prominent in the chloroform extract. Isolation of antioxidant compound therefore from this plant may require the use of intermediate polar solvent. Antioxidant activity of plants is attributed to the presence of phytochemicals mostly flavonoids, which could account for their antioxidant properties (Esimone et al., 2009).

The excision wound healing experiment revealed that the ethanol extracts of *P. lappacea* exhibited wound healing activities with 2.0 and 10.0% w/w PLE exhibiting significant wound contractions of on days 7, 9 and 11 (Figure 4) with reference to the untreated. PLC exhibited wound healing activities with 1.0 and 0.5% w/w PLC demonstrating significant wound contractions on days 7, 9 and 11 (Figure 3) compared to the untreated. The results revealed that the extracts of *P. lappacea* possess wound healing properties with significant wound contractions beginning from day 7. There was extensive fibrosis and formation of collagen tissues resulting in the significant wound contractions experienced from days 7 to 11 particularly for 0.5, 1.0, 2.0 and 10.0% w/w PLE. The histo-pathology of wounds treated with 2.0 and 10.0% w/w PLE depicted collagenation and granulation tissue formation, evident of appreciable healing. Complete wound scar resulted from increased collagenation and tissue

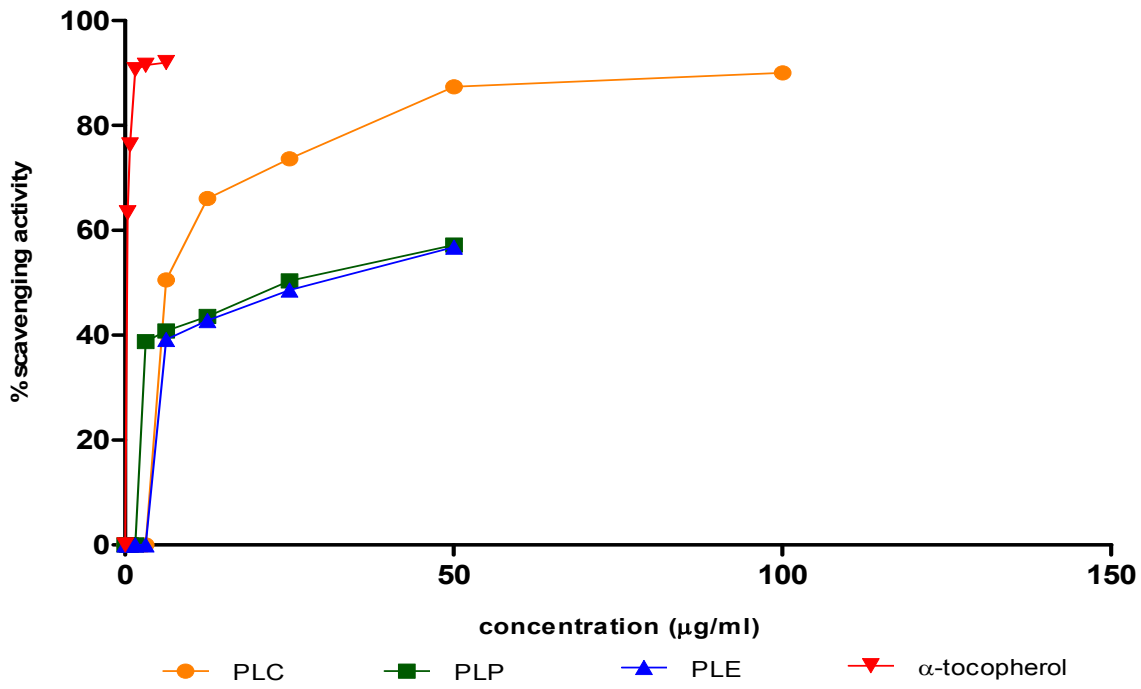

Figure 2. Free radical scavenging activity of extracts of *P. lappacea* and α-tocopherol. PLC = chloroform leaf extract of *P. lappacea*; PLP = pet ether leaf extract of *P. lappacea*; PLE = ethanol leaf extract of *P. lappacea*.

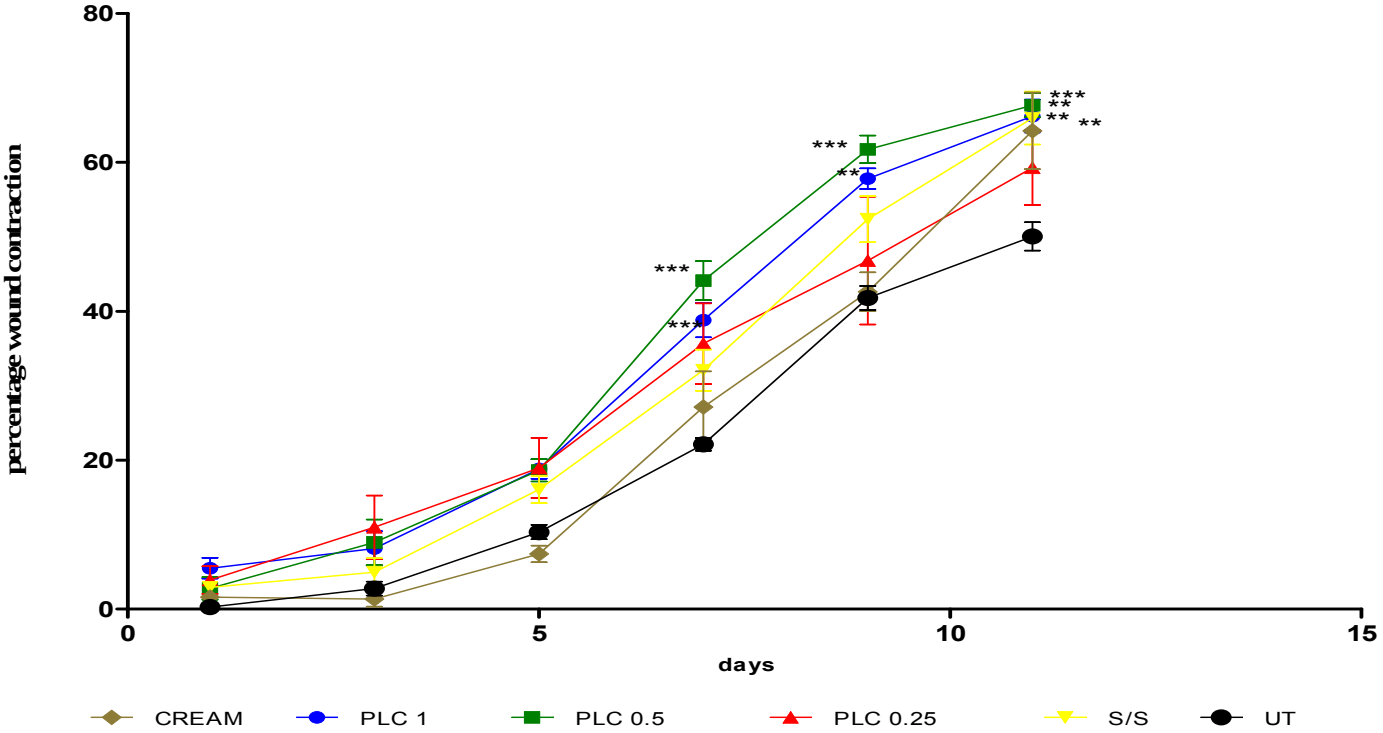

Figure 3. Percentage wound contraction of chloroform leaf extract of *P. lappacea.* PLC= chloroform leaf extract of *P. lappacea*; PLC 1=1% w/w cream; PLC 0.5= 0.5% w/w cream; PLC 0.25=0.25% w/w cream; S/S=silver sulphadiazine; N=5 rats per group; *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant compared with the untreated wounds.

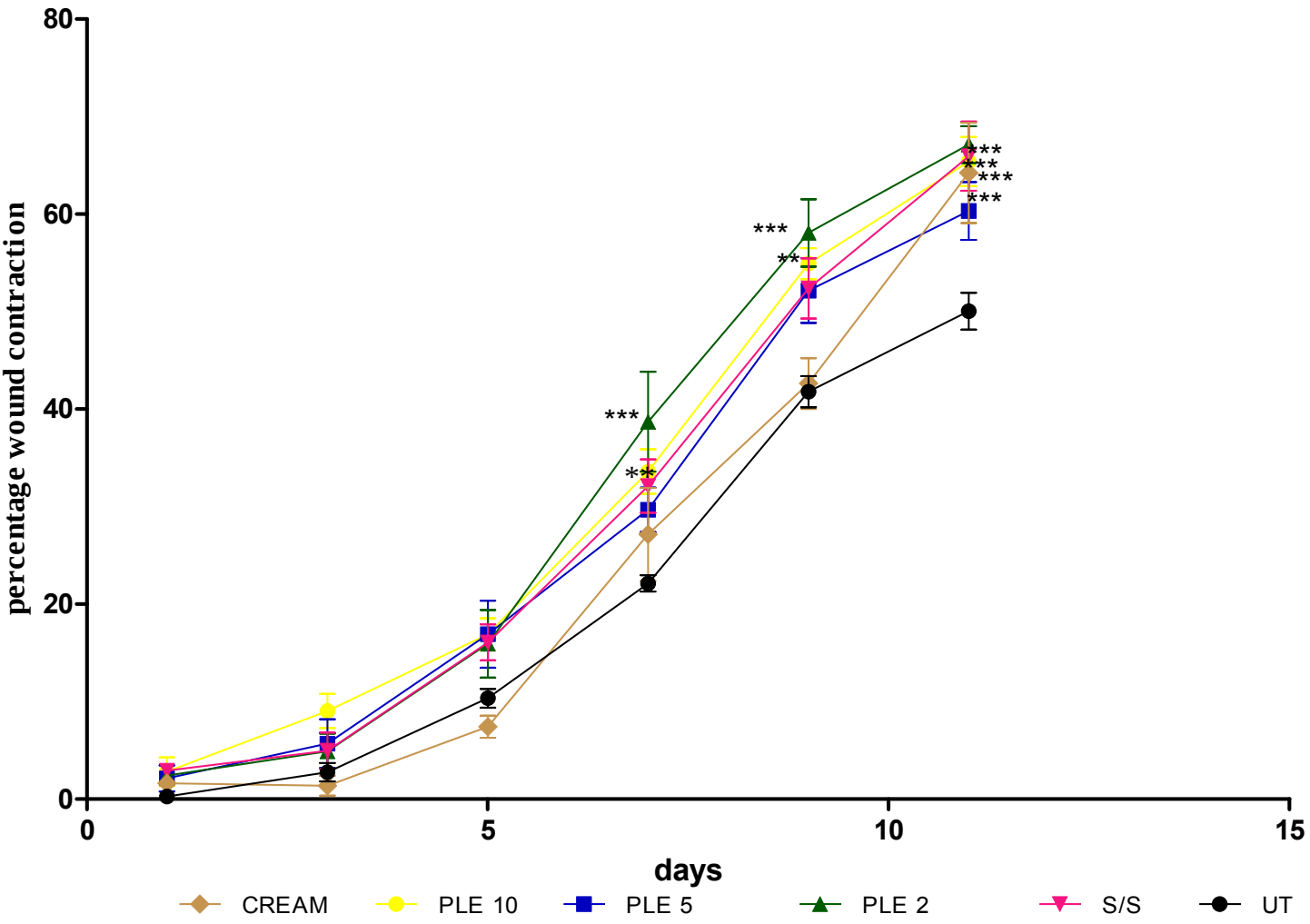

Figure 4. Percentage wound contraction of ethanol leaf extract of *P. lappacea*. PLE= ethanol leaf extract of *P. lappacea*; PLE 10= 10% w/w cream; PLE 5= 5% w/w cream; PLE 2= 2% w/w cream; S/S= 1 % w/w silver sulphadiazine; N=5 rats per group; *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant compared with the untreated wounds.

tissue re-epithelialisation. The untreated wounds as well as wounds treated with the vehicle (aqueous cream base) only exhibited poor wound healing with persistent inflammation and oedema (Figure 5). This indicates that the vehicle had no interference with the wound healing effects of the extracts and that the effects are solely as a result of influence of the extracts. There is a need for the isolation and structural elucidation of the bioactive compounds responsible for the above biological properties.

Conclusion

Ethanol (PLE), chloroform (PLC) and petroleum ether (PLP) leaf extracts of *P. lappacea* exhibited antioxidant properties. The ethanol and chloroform extracts demonstrated good wound healing properties through improved rate of wound closure, collagenation and granulation tissue formation in treated wound tissues compared with the untreated.

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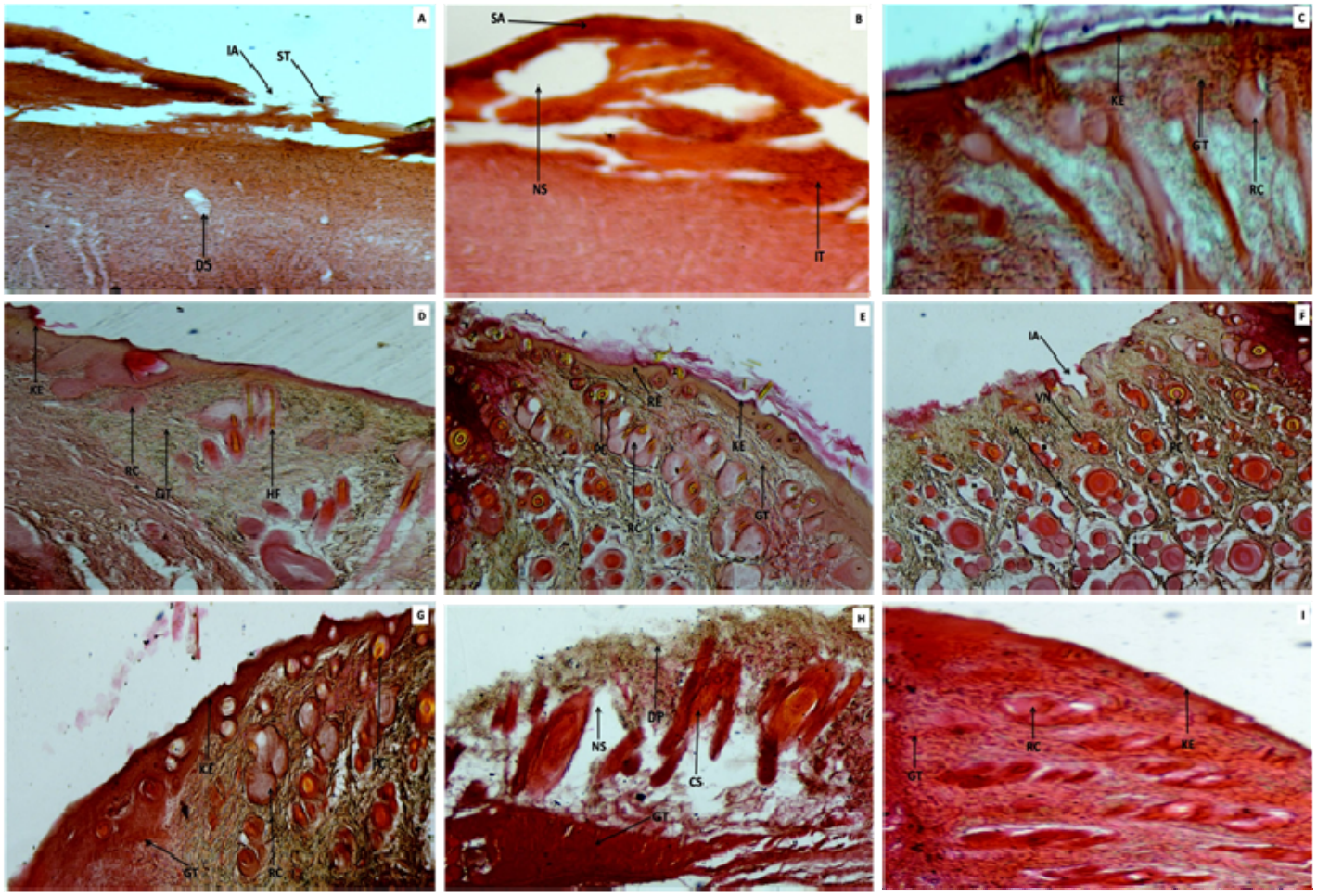

Figure 5. Histopathology of wound tissues. (A) Untreated: Persistent inflammation with marked tissue necrosis. (B) Vehicle: Persistent inflammation and oedema, evident of chronic inflammation, but with appreciable re-epithelialisation as evident by completely healed wound area. (C) Silver sulphadiazine 1%w/w: Granulation tissue formation and angiogenesis with evidence of apoptosis due tissue necrosis with marked re-epithelialisation. (D) PLC 1%: Appreciable angiogenesis and granulation tissue formation with evidence of hair follicle and tissue restitution. Complete scaring resulted from increased collagenation and re-epithelialisation. (E) PLC 0.5%: Dispersed pus cells with increased collagenation and re-epithelialisation, indicative of high rate of healing. (F) PLC 0.25%: Increased fibrosis with profuse angiogenesis and vascularity, indicative of delayed healing. (G) PLE 10%: Increased collagenation and re-epithelialisation, with reduced pus cells, indicative of an appreciable rate of healing. (H) PLE 5%: Highly oedematous tissue with diffused pus evident of persistent tissue necrosis. (I) PLE 2%: Collagenation and granulation tissue formation, evident of appreciable healing. Complete wound scar resulted from increased collagenation and re-epithelialisation.
Note: CS: Collagen strands, DP: Diffused pus, DS: Dead spaces, FM: Fibrin meshwork, GT: Granulation tissue, HF: Hair follicle, IA: Incomplete wound area, IT: Inflamed tissue, KE: Keratinous epithelium, NS: Necrotic spaces, PC: Pus cells, RC: Reconstituted collagen, RE: Reconstituted epithelium, SA: Swollen wound area, ST: Sloughing tissue, VN: Vascular network.

Conflict of interest

Authors declare that they have no competing interests.

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