A study of the antimicrobial andwound healing activities of theethanolic leave extract of *Anchomanesdifformis*(Blume).

ABSTRACT: should be structured

The aim of this current research is to ascertain the antimicrobial and wound healing properties of the leaves *Anchomanesdifformis*(Araceae). Cold maceration method was used for the extraction of the powdered leaves using 96.4% ethanol. Phytochemical analysis was carried on both powdered leaves and the extract to determine the presence of some secondary plant metabolites. Agar well diffusion method was used to determine the zone of inhibition on some microorganism that infect wounds and the MIC of the extract was determined. Excision wound model was used for the wound healing analysis on Wister albino rat. Tannins, saponins, alkaloids, flavonoids, and phenols were present in both the powdered leaves and the extract. Glycoside was present in the powdered leaves however, absent in the yield extract. The *A. difformis* extract demonstrated antimicrobial activity with MICs of >20mg/ml, 10mg/ml, 1.25mg/ml, 0.2679mg/ml for *Klebsiella pneumonia*, *Staph. aureus*, *E. coli*, *P. aeruginosa* respectively. The wound healing results showed a significant ($p<0.05$) dose-depended response when the percentage wound contractions were plotted against the various concentrations of the plant extract formulated as ointment. Percentage wound healing contractions of 83.51%, 90.79%,94.66% and 98.65%were recorded for 2.5%, 5%, 10%, 15% concentrations of *A. difformis* extracts respectively. ~~From the result above~~, *A. difformis* has shown to have antimicrobial and wound healing properties. The study provides justification for the plant's traditional usage to treat infections and wounds.

Keyword: Wounds, medicinal plants, antimicrobial agents, MIC, Agar well diffusion

1. INTRODUCTION

Wound is a disruption of the cellular and anatomical continuity of a tissue (cooper *et al*, 2001). The disruption may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue. Current estimate indicates the 1.51 to 2.21 per 1000 persons worldwidesuffer from chronic wounds(Zhu et al., 2022). Chronic wounds constantly produce inflammatory mediators that induce pain and swelling at the wound site. Chronic wound may lead to multiple organ failure or septicemia which may lead to death of patient (Bowler, 2002).

Chronic wounds represent a significant burden to patients, healthcare professionals and the US healthcare system, affecting 5.7 million patients costing an estimated $20 billion annually. To effectively manage these problems, one must understand the normal healing process and engineer a healthy physical and biochemical environment (Sen et al., 2009).

The use of medicinal plants to treat infections and diseases has been a practice since the inception of life (Borchardt, 2002). Africa is endowed with vast and diverse vegetation; making medicinal plants very accessible (Agyare et al., 2016). It has been revealed that approximately 80% of the people of Africa use therapeutic plants and herbs to management their infections and conditions; malaria, diabetes, chronic wounds and skin ulcers due to their affordability and accessibility(Korsah et al., 2021). The traditional medicine has a long history of use and constitutes an important aspect of the culture and life of Africans (Builders & Builders, 2016). The aim of treating a wound is to either shorten the time or to minimize the undesirable consequences(Raina et al., 2008). Medicinal plants are enriched with bioactives which act synergistically to stimulate wound healing. These bioactives are able to accelerate the proliferation and differentiation, prevent microbial contamination and enhance re-epithelization of the broken skin tissues. Medicinal plants are also able to prevent oxidative stress caused by reactive oxygen species that may have implication on wound healing. Plants documented to have wound healing properties includes *Aloe vera*, *Vinca rosea, Rosemarinus officinalis*, *Camellia sinensis*, *Carrica papaya and Moringa oleifera* etc. *Curcuma domestica*, *Daucus carota*, *Emblica officinalis*, *Glycyrrhiza glabra*, *Mangifera indica*, *Allium sativum*, *Momordica charantia*just to mention feware medicinal plants documented to have antioxidant properties (Agyare et al., 2016; Guetchueng et al., 2015; Zakian et al., 2022)

Anchomanesdifformis(Blume)Engl.Pallidus,commonlyknownasforestAnchomanesisaplant ofthefamilyAraceae.*Anchomanesdifformis* is distributed widely in wetlands and terrestrial areas ofwest tropical Africa including Nigeria, Ghana, Ivory Coast, Sierra Leone, Senegal and Togo(Ahmed, 2018). According to study by (Agyare et al., 2016)*Anchomanesdifformis*, possesses antimicrobial, anti-inflammatory andantioxidant properties. *Anchormanesdifformis*(Araceae) is made up the following parts; root, rhizome, and stem and leaves just as most plant. The leaf has three (3) main divisions with each leafletmeasuring 10cm long by 8 cm wide, an olive-green petiole and dull darkish-brown spot at the base of the short and rigid prickles. It has green stout prickly stem. The tuber is harvested from the wild as an emergency food in times of need. It is a multi-purpose plant and each partof the part has a medicinal property. Folklorically, theroots are used to treat cough, diabetes, dysentery and throat infections(Oyetayo, 2007). The stem and rhizomeare used to ease child birth, constipation, hernia, kidney pain, abdominal pain and treat diabetes, gonorrhea, asthma, epilepsy etc. More importantly, the leaves are used as galactagogue, antitussive, purgative and for wounds and minor cuts (Oghale & Idu, 2016). Indigenes using *Anchomanesdifformis*apply directly into their wound as a poultice ~~which~~. Herbal products intended to be used as wound healing products must be scientifically validated (Krishnan, 2006). In view of this, this research work is to investigate antimicrobial properties and the wound healing activities of the leaves of *Anchomanesdifformis.*

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Chemicals Used: Ethanol 98%v/v (GPR, BDH, Poole, UK.), chloroform 99.9%v/v (AR, Marek, UK.), normal saline infusion 0.9%w/v (Kabi Pot. Ltd. Pune, India.), Fehling's solution A and B (GPR, BDH, Poole, UK), Sulphuric acid 98.5%v/v (GPR, BDH, Poole, UK), Hydrochloric acid 36%v/v (GPR, BDH, Poole, UK), Sodium hydroxide 96%v/v (GPR, BDH, Poole, UK), Dragendorff's reagent 50%v/v (AR, Marek, UK), Ammonia solution 30%v/v (AR, Marek, UK) and Iron (II) chloride 97%v/v (GPR, BDH, Poole, UK).

2.1.1 Reference Compound: Amoxacillin(for antimicrobial assay) and Drez ointment (for the wound healing test).

Instruments and equipment/Glass ware: Rotary evaporation-R 10 (Buch, Germany), Hot air oven (Sanyo, OMT Oven, Gallenkamp, UK), Gallenkamp Plus II Cooled Incubator (Gallenkamp, UK), Thermostatically controlled water bath (R76 New Brunswick, Edison N.J, USA Electronic weighing balance (Ohaus corporation, Pine Brook, N.J, UK), No. 5 Cork borer (Gerber Instruments, AG, Holland), Portable autoclave (Basildon, Ltd., UK), 500ml Separating funnel (GMBH, Wertheim, Germany), Beakers50ml, 250ml, 500ml and 1L (GMBH, Wertheim, Germany), Test tubes (GMBH, Wertheim, Germany), 1ml, 10ml dropping pipette (GMBH, Wertheim, Germany), 250ml,500ml conical flask (GMBH, Wertheim, Germany) and Petri dish (GMBH, Wertheim, Germany).

2.1.2 Test Organisms: *Escherichia coli (*ATCC25922*), Staphylococcus aureus (*ATCC26923*), Pseudomonas aeruginosa (*ATCC27853*), Klebsiella pneumonia (*ATCC-BAA 1705)

2.2 Plant collection

The leaves together with the stems of *Anchomanesdifformis*(Blume) were collected from Adansi North (Asokwa junction) in Ashanti Region. The collected specimen was authenticated by Miss Miriam Tagoe, the head of the School of Pharmacy Herbarium, and voucher number (CUC/A/NK/008)was deposited in the Department's Herbarium. All dried plant materials were milled to coarse powders with mechanical milling machine and stored in airtight amber glass containers and well covered.

2.3 Preparation of the Plant

The procedure of (Adebayo et al., 2014)was employed for the processing and extraction. The fresh leaves were dried under shade in the pharmacognosy laboratory, and were milled into powder. The obtained powder was stored in a dry container.

2.4 Preparation of animals

Wistar albino rats (males and females) of weight between 150-170g were obtained from Noguchi Memorial Institute for Medical Research (NMIMR), Ghana. These rats were kept in stainless steel cages at the animal house,Department of Pharmacology, Central University, under ambient temperature (25°C), light, and relative humidity (55 to 60%). The rats were maintained on

standard pellet diet and water daily for one week prior to the experiments for acclimatization at room temperature.

2.5 Ethical approval

Rats were handled according to guidelines for care and use of laboratory animals laid down by the National Institute of Health (NIH, Department of Health and Human Services Publication No. 5, Revised 1985)(Information & Branch, 2001). All animal studies in this research were approved by the Animal Ethical Committee at the School of Pharmacy, Central University, Accra, Ghana.(Ethics approval letter no:…)

2.6 Extraction of the leaves of the plant

The powdered material (430.56g) was extracted using 96.4% ethanol solvent by cold maceration method of extraction. The extracts were decanted severally until no further green coloration of the extract was observed upon addition of ethanol. The collected volume of the ethanol extract was concentrated using rotator evaporator and a deep green mass of weight 41.89g obtained. The ethanolic *Anchomanesdifformis*extract was coded as ADC.

2.7 Phytochemical screening

Phytochemical components of *Anchomanesdifformis*(Blume) leaf extract was identified using conventional techniques outlined by (Evans, 2009).

2.8Antimicrobial studies

The leave extract of *Anchomanesdifformis*(Blume) was investigated for the antimicrobial activity against*Klebsiella pneumonia, staphylococcus aureus, pseudomonas aeruginosa and Escherichia coli* using agar well diffusion method and the zone of inhibition was measured(Holder & Boyce, 1994).

2.8.1 Sterilization of Materials

All glassware, test tubes and petri dishes were washed with detergents and rinse in distilled water properly. These items were air dried and then sterilized in autoclave at 121 degrees Celsius for 15 minutes. Nutrient agar was prepared according to (Pharmacopoeia, 2013)andsterilized in autoclave at 121 degrees celsius for 15 minutes. Cork borer, glass rods and forceps were sterilized by dipping in 70% ethanol which was then flamed in Bunsen flame. The inoculating loop was also sterilized by heating to redness using naked flame before and after each use as described in(Yadav et al., 2015).

2.8.2 Methodology:

The antibacterial activity of the leaf extracts were carried out using agar well diffusion and a modifiedmethoddescribed in(Yadav et al., 2015). Organisms known to affect wounds such asGram positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia*) were selected. The microorganisms were seeded on Muller–Hinton plates and then incubated in a bacteriological oven at 37 ± 1^{0}C for 24

hours. Bacterial suspension (inoculum) was diluted with sterile physiological solution to 0.5 McFarland barium sulfate standard (1×10^8 CFU/ml). Concentrations of 2.5, 5.0, 10.0 and 15.0 mg/ml in 200 µl volume were prepared with the aid of a micropipette into the plates.The zones of growth inhibition were measured after 24 hours incubationat 37^0C. The antimicrobial activities of the extracts were compared with the activity of a standard antibiotic, amoxicillin (1 mg/ml). Experiments were carried out in triplicate, and the antibacterial activity was expressed as the mean of the inhibition diameters (mm) produced. Amoxicillin was used as standard. Zones of inhibition was examined, measured (mm) and recorded.

2.8.3Minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of the extracts and the standard drug were evaluated using a modified solid media techniquedescribed in(Wiegand et al., 2008).The minimum inhibitory concentration was calculated from the zones of inhibition by a graph method. The zones of inhibition were plotted against the log concentration on a graph sheet.The line of best fit was extended to intercept with the x axis (log concentration). The MIC was calculated as the antilog of x. This was repeated for all the test organisms.

2.9Wound Healing Studies Using Excision Wound Model

The wound healing analysis was carried out by methods described by (Chitra et al., 2009). Excision wound model was used to evaluate the rate of wound contraction and epithelization. Thirty-fivewistar rats of both sexes with body weight 120-170g were used for this study. The rats were fed with standard feed and clean water. The rats were divided into six groups of five rats as indicated on Table 1. The rats were shaved and anesthetized with ketamine (30mg/kg, ip). Approximately 2cm wound area of was marked on the back of the ratsusing marker filled with ammonium oxalate violet paint. The marked shaved skin zonewas then cut carefully in its thickness using razor blade and cleaned with 70% ethanol. The created wounds were allowed to assume it normal size as the tissues stretches and the initial wound size was taken and recorded using digital caliper. Aqueous creams used for the wound healing experiment were prepared according to the method described inthe British Pharmacopoeia (2013). Preservatives were excluded from the creamto prevent its interference with the wound healing activity of the extracts.Aqueous cream (100g) was prepared by mixing 30 g of emulsifying ointment in 70 mL of sterile distilled watermaintained in a water bath maintained at temperature 60 ^{0}C. The mixture was then stirred until it was melted and allowed to cool. Different concentrations of *Anchomanesdifformis*extract (i.e., 2.5 % w/w; 5.0 % w/w; 10 % w/w; 15.0% w/w) were incorporated into the aqueous cream and kept in a labelled ointment container. The physical stability of the creams was monitored for phase separation, colour, odour, and texture. Wound treatment began 24 hr post-injury and lasted for 14 days. The rate of wound contraction calculated as given in the formula below:

$$\%\ wound\ contraction = \frac{Healed\ \ area}{Original\ \ wound\ \ area} \times\ 100$$

(Healed area = original wound area − present wound area)(Murthy et al., 2013).

Table 1 Grouping of rats for wound healing treatment

Groups	Description
	14 days treatment
I	2.5% *A. difformis* extract
II	5% *A. difformis* extract
III	10% *A. difformis* extract
IV	15%*A. difformis* extract
V	Drez ointment (PC)
VI	Untreated (0.9% sodium chloride) NC

PC= Positive Control, NC= Negative Control

Plate 1.Picture showing the excision wound process

3.0 RESULTS

In the phytochemicalscreeningtannins, saponins, flavonoids, alkaloids and phenols werepresent in both the powdered plant material and plant extract. Glycosides was present in thepowderedplantmaterialand absentintheplantextract as seen on table 2.Antimicrobial assay was carried out on the extract as a preliminary test to determine theconcentrations that would be used in the preparation of the ointment and also to confirm theantimicrobialeffect of *Anchomanesdifformis*.Sensitivitytest:agarcupplate methodused.The zones of inhibition were

measured and recorded as shownintable2. MIC of the extracts, which measure the minimum concentration of the extracts to cause inhibition in a microorganism were also evaluated as seen on table 3.The excision wound model was employed. Both treated and untreated groups were analyzed for a period of 14 days. Figure 1,2 and 3 have the results from the wound healing assay.

3.1 Phytochemical analysis

Table 2: Results of phytochemical analysis of the dried leaves powder and the extract of *A. difformis*

Phytochemical test	***AnchomanesDifformis* dried leaves powder**	***AnchomanesDifformis* yield extract**
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Phenols	+	+
Alkaloids (Wagner test)	+	+
Glycoside	+	-

Key: Present = (+) and Negative = (-)

Table 3: Zone of inhibition of the extracts and reference drug.

Microorganism	**Concentration/ Zone of inhibition (mm)**				
	Amoxicillin (mg/ml)	**20mg/ml**	**10mg/ml**	**5mg/ml**	**2.5mg/ml**
P. aeruginosa	14 ±06	11 ±45	10 ±04	7 ±43	6 ±03
Staph. aureus	18 ±08	6 ±76	-	-	-
E. coli	5 ±03	8 ±23	5 ±09	4 ±12	2 ±09
Klebsiella pneumonia	5 ±11	-	-	-	-

Table 4: MIC result obtained for microorganism (*Pseudomonas aeruginosa, Escherichia coli, Staph. aureus, and Klebsiella pneumonia)*

Organism	MIC (mg/ml) of ADC	MIC of Amoxicillin (mg/ml)
P. aeruginosa	0.2679	< 0.5
E. coli	1.25	< 0.5
Staph. aureus	> 10	< 0.5
Klebsiella pneumonia.	>20	< 0.5

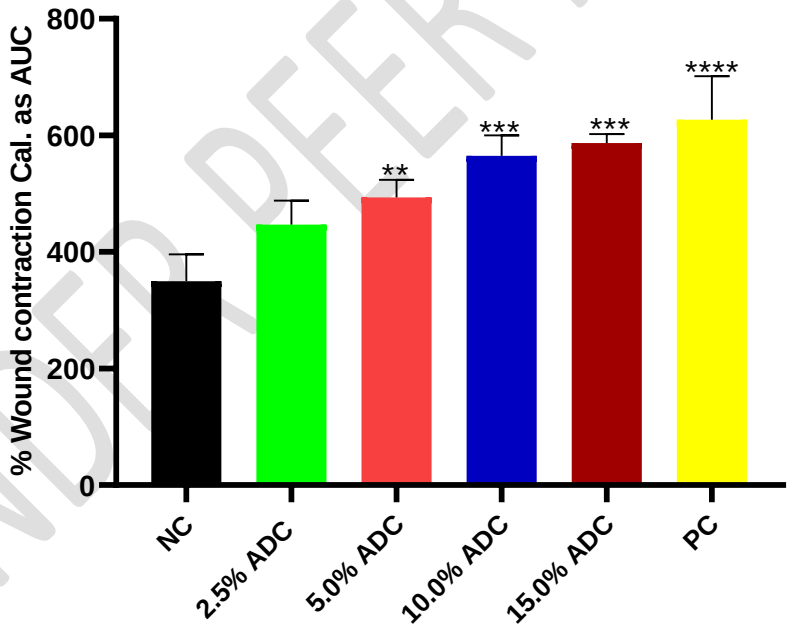

Fig 1.0 This represents the wound healing response (AUC) using Wister albino rat.Thedataisexpressedasmean+/-SED(n=5),**p<0.05(i.e. p=0.0065)ascomparedwiththe0.9% sterile sodium chloride solution (negative control). PC (positive control); Drez ointment. One way ANOVA was used forthisanalysisfollowedby Dunnet'smultiple comparison.

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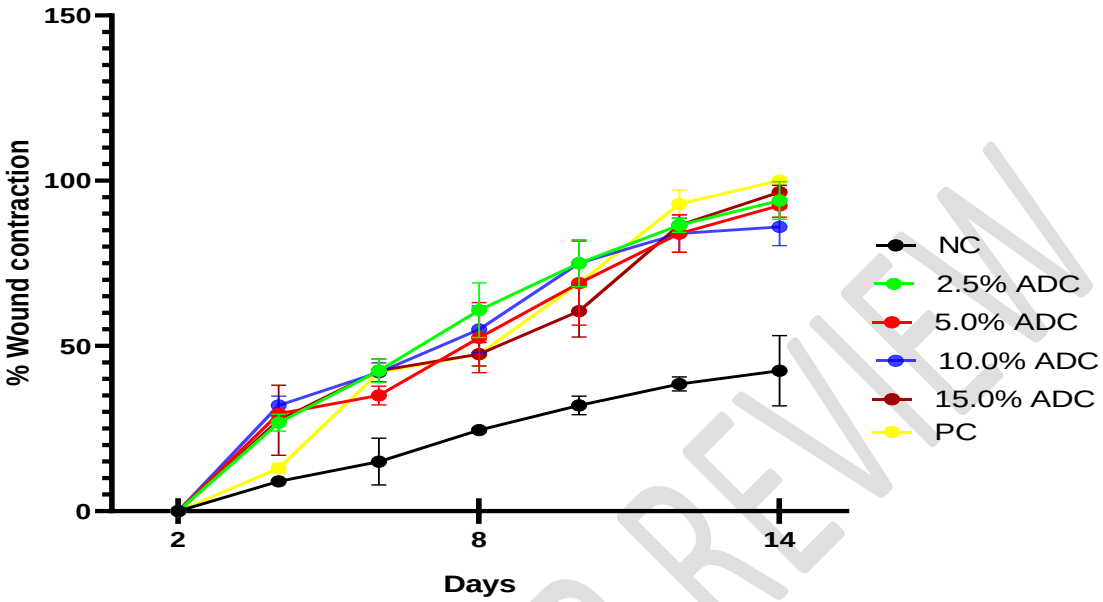
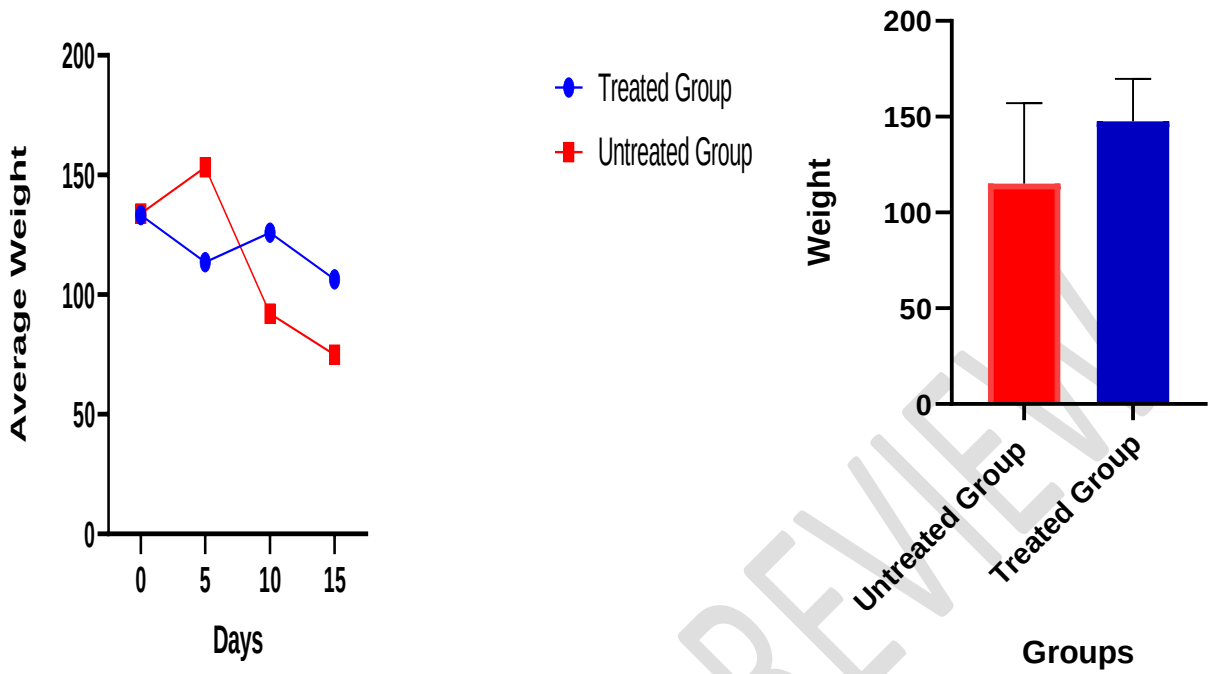


Figure 2 : Represents the time course for the healing and percentage wound contraction. The data is expressed as mean+/-SED (n=5), **p<0.05 (i.e. p=0.0065) as compared with the 0.9% sterile sodium chloride solution (negative control). One way ANOVA was used for this analysis followed by Dunnet's multiple comparison.



(A)

Fig.3(A) Average weight of rats over 15-day study period and (B) average weight treated and untreated groups on day 15

4.0 Discussion

Wound healing is a natural phenomenon which arise from wound contraction(Agyare et al., 2009; Agyare et al., 2016; Wasman et al., 2010). Usually wound contraction begins with proliferation phase which covers angiogenesis, collagen deposition, fibroblast proliferation, granulation, tissue formation, epithelialization, and wound contraction. As the wound contract, it leads to the restoration of the lost and damaged cellular structures and epidermal tissue layers Plant in general have played an important role in wound healing and in management of diverse clinical conditions. Plants have been known to contain important metabolites that is responsible for their pharmacological activity they exhibit.

The pharmacological prowess of a plant is a reflection of its bioactive constituents (Korsah et al., 2021).The phytochemical analysis performed on *A. differmis* showed the presence of major secondary plant metabolites such as alkaloids, tannins, saponins, flavonoids, and tannins both on the extract and the powdered samples. Additionally, glycoside was present in the powdered samples however, it was absent in the extract. (Adeyemi et al., 2015)indicated that concentration of ethanol has significant effect in plant extraction. The lower the ethanol concentration the higher and better the glucosidal yield in the extract. This phytochemical are responsible for

various pharmacological properties exhibited by plant extract. Some plant metabolites such as flavonoids, tannins and saponins are known to promote wound healing. Flavonoids help in dwindling the lipid peroxidation which causes necrosis and reduces angiogenesis. Tannins, on the other hand have, antimicrobial and antioxidant which is crucial in improving and promoting wound healing. Saponins are known to augment the immune status to facilitate healing. The wound healing contraction observed may be due to the ability of some phytochemicals in the creams that caused collagen expression, its subsequent maturity, angiogenesis which increased wound tensile strength during the proliferation phase of wound healing(Al-Kadmi, 2012; Lei et al., 2019; Mahmood et al., 2010; Zirwa Anwar & Fatma Hussain, 2017)

The susceptibility pattern of *A. differmis* was determined using the zone of inhibition(which is the clear zone around each well of the agents) for the various test organisms. There wasa linear relationship between the extract dose and the susceptibility pattern of the test organisms. The highest concentration recorded the highest zone of inhibition as seen Table 3. *A. differmis* was more sensitive in *Pseudomonas aeruginosa*and *Staphylococcus aureus.* The *A. differmis*was found to have an inhibitory effect on *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* with MICs of, 0.2679mg/ml, 1.25mg/ml, >10mg/ml and >20mg/ml respectively. Saponins and alkaloids have been known to possess selective antibacterial properties. This antibacterial effect observed might be due to presence of saponins and alkaloid present in the plant as shown on Table 2(Ayensu & Quartey, 2015).

A. differmis extract exhibited a significant ($p<0.05$) dose depended wound healing response, as the percentage wound contraction increased with increasing concentration of the *Anchomanesdifformis* extract. The respective percentage wound contractionswere 83.51%, 90.79%, 94.66%, and 98.65% for 2.5%, 5%, 10% and 15% concentrations of aqueous cream of *A.differmis*.Wound healing generally has the following phases; Hemostasis, Inflammation, Proliferation and Remodeling. In the inflammation phase, proliferation of inflammatory mediators (neutrophils and monocyte etc.) are triggered(Al-Kadmi, 2012; Muhammad & Muhammad, 2005). Inflammation can be classified as mild, moderate and severe (excessive) based on the level of circulating cell types and quantity. Various wound healing studies have proposed that the inflammatory phase has an effect on the final healed wound. A moderate inflammation is vital for wound healing. However, an excessive inflammation normally results in scaring. *A. differmis* extract healed wounds had neither scars nor hypertrophic scar. It therefore imperative to state that the *A. differmis* extract caused no pain to the rats upon application of the formulations because there was no sign of restlessness and scratching of the wound site on extract application. The extract was able to enhance the feeding ability of the test rats and therefore causing an increase in their overall weight (fig 3).

Limitation of the study ????

5.1 Conclusion

This research reveals that *Anchomanesdifformis* extract has antimicrobial activity against *P. aeruginosa*, *E. coli*, *Staph. aureus*, and *Klebsiella pneumonia*, wound healing. *A. difformis* extract can therefore be used in wound where antimicrobial and antioxidant agent are required.

Elaborate and clarify conclusion part ..

Data Availability

The data used to support the findings of this study are included in the article and also available from the corresponding author upon request.

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