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Assessment of the Wound Healing Property of the Ethanolic Seed Extract of *Eleusine coracana* Linn using the Chorioallantoic Membrane Model

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Access to readily available material for wound healing is very important. The wound healing property of *Eleusine coracana* a food source in Nigeria was therefore investigated. The ethanolic crude extract of the seed flour was prepared using maceration and qualitative phytochemical screening was carried out on the crude extract using standard methods. The antimicrobial activity of the *E. coracana* extract was determined using the disc diffusion method against *E. coli, S. aureus, P. aeruginosa, and C. tetani*. The wound healing property assay was carried out using the Chorioallantoic Membrane Model (CAM) at 50mg, 100mg, 150mg, and 200mg/ml concentration with the basic Fibroblast Growth Factor (bFGF) as the positive control. The crude extract was further fractionated into Hexane, Methanolic, and Aqueous fractions and then subjected to amino acid content analysis using the Applied Biosystems Phenylthiohydantoin (PTH) Amino Acid Analyzer. The phytochemical screening reveals the presence of alkaloids, steroids, terpenes, flavonoids, carbohydrates, glycoside, and protein. *E. coli and S. aureus* were susceptible to the *E. coracana*

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extract at a Minimum Inhibitory Concentration (MIC) of 50µg/ml while the *P. aeruginosa* was only susceptible at 200µg/ml and *C. tetani* was not susceptible at all the concentrations used. An increase in percentage angiogenesis was observed at all doses of the extract used indicating good wound healing properties. The amino acid profile reveals the presence of glycine, a proline that is important in wound healing. The *E. coracana* seeds, therefore, have wound healing properties based on the CAM model.

Keywords: Angiogenesis; wound healing; E. coracana; antimicrobial; amino acid.

1. INTRODUCTION

Current estimates indicate that nearly 6 million people suffer from chronic wounds worldwide [1,2]. Non-healing or chronic wounds result in enormous health care expenditures, with the total cost estimated at more than $3 billion annually [1,2]. Over the last decades, the search for newer and potent agents from nature (plants, marine environment, fungi, and other microorganisms) to manage chronic wounds especially, in patients with underlying metabolic disorders has increased immensely [3,4].

To identify cheaper alternatives that serve as a possible food supplement that can improve wound healing from readily available staple food, this study seeks to verify the wound healing properties of finger millet (*Eleusine coracana* Linn) seeds extract using the Chorio-Allantoic Membrane assay. Finger Millets (*Eleusine coracana* Linn), is a member of the cereal family and also serves as a good source of food for most of the population of developing countries [5]. It also has some folklore claims of certain medicinal uses especially in Nigeria [6]. Since the role of protein has been established in wound healing an assessment of the amino acid content of its seed extracts will also be carried out.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Two kilograms (2kg) of the plant material finger millet (*Eleusine coracana* Linn) seeds were properly identified and weighed. It was then washed, air-dried washed with distilled water, and powdered into flour using a clean dry multiple-pronged electric blender.

2.1.1 Extraction of the seed flour

Two (2) kilograms of the powdered seed flour was soaked with 3L of ethanol in a 10L container for 72 hs at room temperature with intermittent shaking. It was then filtered and the filtrate concentrated with a rotary evaporator at 40°C.

2.1.2 Fractionation of the crude extract

The ethanolic crude extract of the *Eleucine coracana* seeds was further fractionated in an open column packed with silica gel (F24) and then eluted with Hexane, Ethyl acetate, Methanol and Water consecutively to yield the Hexane, Ethyl acetate, Methanol, and Aqueous fractions respectively.

2.2 Qualitative Phytochemical Screening

A test for triterpenoid, glycosides, alkaloids, carbohydrates, saponins, flavonoids, protein, anthraquinones, steroids, and tannins was carried out on the crude extract and the respective fractions using standard methods described by Shaikh, & Patil, [7] and Harbone, [8].

2.3 Amino Acid Analysis/Profiling

The Amino Acid profile in the known sample (Standard), was determined using methods described by Benitez (1989). A 500mg of the standard was defatted using chloroform/methanol (2:1), hydrolyzed, evaporated in a rotary evaporator, and a 60µL of the hydrolysate loaded into the Applied Biosystems PTH Amino Acid Analyzer. The hydrolysis was carried out by the use of 7ml of 6N HCl and the sample in a glass ampoule which was then sealed and incubated at $105^0C \pm 5^0C$ for 22 hs. The content was then filtered and evaporated to dryness using a rotary evaporator. An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids which are used in calculating the amino acid content of the sample. This procedure was repeated using the four fractions Hexane, Ethyl acetate, Methanol, and Aqueous fractions.

2.4 Percentage Nitrogen Determination

A small amount (115mg) of ground sample was weighed, wrapped in Whatman filter paper (No.1), and put in the Kjeldahl digestion flask. Concentrated sulphuric acid (10ml) was added. Catalyst mixture (0.5g) containing sodium sulfate (Na_2SO_4), copper sulphate ($CuSO_4$), and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Six pieces of anti-bumping granules were added. The flask was then put in the Kjeldahl digestion apparatus for 3 hs until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100ml in a standard volumetric flask. Aliquot (10m1) of the diluted solution with 10ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70ml of distillate was collected.

The distillate was then titrated with standardized 0.01 N hydrochloric acid to a grey-colored endpoint. The nitrogen content was then calculated using the formula;

$$Percentage\ Nitrogen = \frac{(a * b) * 0.01 * 14 * V * 100}{W * C}$$

[a = titre value of the digested sample; b = titre value of blank sample; v = volume after dilution (100ml); W = Weight of dried sample (mg); C = Aliquot of the sample used (5ml); 14 =Nitrogen constant in mg.]

The percentage Nitrogen content was utilized in calculating the amino acid content of each sample using the formula:

$$Concentration\left(\frac{g}{100gProtein}\right) = NH * Width@\frac{NH}{2} * Sstd * C$$

Where $Sstd = NEstd * Mol.Weight * \mu AAstd$

%N (Fat Free) =3.20; Volume loaded: 60µL; Dilution = x 5; C=0.009876543

$$C = \frac{(Dilution * \frac{16}{SampleWt(g)} * N\% * Vol.Loaded)}{NH * W(nleucine)}$$

NB: nLeucine is the internal standard

2.5 Antimicrobial Activity Assay (Disc diffusion method)

A well-dried nutrient agar plate was seeded with 1 x 10^6 CFU/ml of test organisms (*E. coli, S. aureus, P. aeruginosa, C. tetani*.). The excess broth was drained and seeded plate dried. Each of the seeded plates was divided into six sections representing the four concentrations of the crude extract of *E. coracana* and the solvent control (water) and standard drug control. Autoclaved filter paper discs were introduced into each concentration of each sample as well as the solvent control. Impregnated discs with samples were placed on the seeded plate in the corresponding position on the already labeled agar plates. Standard commercial discs were used as control while discs impregnated with sterile distilled water were used as the negative control. The plates were incubated at 37^{o}C for 24hrs after which results were read by measuring zones of inhibition in mm. Four organisms were used for the assay namely *E. coli, S. aureus, P. aeruginosa, C. tetani*. The crude extract was prepared in 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml concentrations.

2.6 Chorio-Allantoin Membrane (CAM) Assay

Nine-day-old embryonated eggs were obtained from the Poultry Division, National Veterinary Research Institute (NVRI), Vom. The eggs were candled in the laboratory to ascertain their viability and fitness for use. Viable eggs had their airspace marked out with a pen. The basic Fibroblast Growth Factor (bFGF) gel (commercially obtained from Bharat Biotech Int'l Ltd, Hyderabad, India, serial number 092, S8968 5204 5951), was used as positive control while Phosphate Buffered Saline was used as a negative control. A 2g of the dried plant extract was appropriately weighed and diluted to prepare final concentrations of 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml. Methylcellulose discs were prepared and transferred into sterile empty universal discs. The discs were allowed to absorb the controls and samples for over two (2) hs. The eggs were labeled according to the concentrations of extracts to be treated with.

The shell around the marked area of the identified airspace on the embryonated egg was carefully opened using a sterile pair of scissors after an initial incision without tampering with the chorio-allantoin membrane (CAM). Blood vessels were identified and the impregnated discs were

put along branched vessels according to initial labels. Airspaces were sealed using a transparent adhesive tape and eggs were incubated at 37°C in a wet chamber for 72hs. Results of angiogenesis were read at 0h, 24hs, 48hs, and 72hs from eggs with live embryos.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The ethanolic extract had flavonoids, tannins, carbohydrates, proteins, cardiac glycosides, steroids, and alkaloids. In this extract, saponins, anthraquinones, and terpenoids were completely absent as seen in Table 1. This corresponds with the work done by Singh and Naithani in [9], except for alkaloids that were absent and terpenoids present in the extract. This disparity could be due to the species of finger millet, geographical disposition, and seed morphology. A similar screening of phytoconstituents was carried out for the fractions resulting from a column chromatography of the ethanolic extract of *Eleusine coracana* Linn. Using the polarity index, the hexane, ethyl acetate, ethanol, methanol, and water fractions contain flavonoids, tannins, and cardiac glycosides. The most polar ethanol, methanol, and water fractions contain alkaloids and proteins. The non-polar hexane and midway polar ethyl acetate contain terpenoids and steroids.

3.2 Antibacterial Activity

Except for *Pseudomonas aeruginosa*, all the other organisms were susceptible to 200mg/ml of the extract. On the *Escherichia coli* plate, the zones of inhibition were seen to be dose-dependent. The zones of inhibition showed a corresponding increase with those of the extract. The zone of inhibition shown by the standard disc was eminent and those of the graded extract as well, as shown in Table 2. On the *Staphylococcus aureus* plate, the zones of inhibition were seen to be dose-dependent, with corresponding zones of inhibition increasing with an increase in strength of the extract.

A striking observation was that the zone of inhibition for the standard in *S. aureus* is less than that seen in 100mg/ml, 150mg/ml, and 200mg/ml of the extract. This is likely so because most microorganisms have different metabolic characteristics. Different microbes that belong to various types of microbial metabolisms have been studied for susceptibility by plant extracts. But among them, the group of chemoorganoheterotrophs (an organism that obtains carbon, energy, and reducing equivalents needed for several biosynthetic reactions from organic compounds) is commonly studied for susceptibility testing like *Staphylococcus aureus* [10], as seen in Table 2.

The antimicrobial assay for *Pseudomonas aeruginosa* revealed that the graded doses of the extract showed no zone of inhibition. The standard however showed a clear zone of inhibition. This could be due to differences in cell wall characteristics, and this could also contribute to the anti-microbial susceptibility profile of plant extracts. This differs from the work done and reported by Banerjee *et al*. in [11]. In this case, the procured organism could be a resistant strain or even a peculiar one with specific cell wall characteristics.

On the *Clostridium tetani*, the graded doses of the extract showed no inhibition of bacterial growth except for 200mg/ml and the standard. The measurement of the corresponding zones of inhibitions is as captured in Table 2. The inhibition at the higher dose and that of the standard on this gram-positive organism could be dose-dependent and the metabolic and biochemical characteristics of the organism, especially that gram-positive organisms require specificities in handling (Hugo and Russels, 1998).

3.3 CAM Assay

This is to bring a merger between the established physiological processes of wound healing [12] and angiogenesis [13] on the blood vessels lining the chorioallantoic membrane (CAM) of the embryonated chick [14]. The results were expressed as percentage angiogenesis, which relates the number of new or dilated blood vessels to the vessels treated with the physiological solution, in this case, Phosphate Buffer saline (PBS) provides the percentage increase in blood vessels. Standards of the basic Fibroblast Growth Factor (bFGF) are used, as described by Barua *et al*. in [15], Gupta *et a*l. in [16], and Rupesh *et al*. in [14]. However, the percentage increase in blood vessels was used to express the results of angiogenesis.

The extract was graded, impregnated in commercially purchased methylcellulose discs accordingly then dropped at the junction of a prominent vessel in the airspace of embryonated egg throughout 48hs.

Table 1. Qualitative Phytochemical screening data of *E. coracana* crude extract and fractions

Test	Hexane	EA fraction	EtOH	MeOH	H_2O
Alkaloids	-	-	++	++	+
Saponins	-	-	-	-	-
Tannins	+	+	++	+	+
Flavonoids	+	+	+++	+++	+++
Carbohydrates	-	-	+	+++	++
Proteins	-	-	++	++	-
Terpenoids	+	+	+	-	-
Cardiac glycosides	+	+	+	+	+
Anthraquinones	-	-	-	-	-
Steroids	+	+	+	++	-

Key: - = absence, + = slightly present, ++ = present, +++ = significantly present

Table 2. Antimicrobial activity of the crude extract

S/N	MIC (mcg/ml)	Zones of inhibition (mm)			
		E. coli	***S. aureus***	***P. aeruginosa***	***C. tetani***
1	Solvent	0	0	0	0
2	50	10.4	7.9	0	0
3	100	11.1	8.4	0	0
4	150	11.9	12.1	0	0
5	200	12.1	13.8	0	18.0
6	Standard	15.8	8.1	26.4	18.4

Standasrds: E. coli = ATCC 25922; S. aureus = ATCC 25923; P.aeruginosa = ATCC 27853

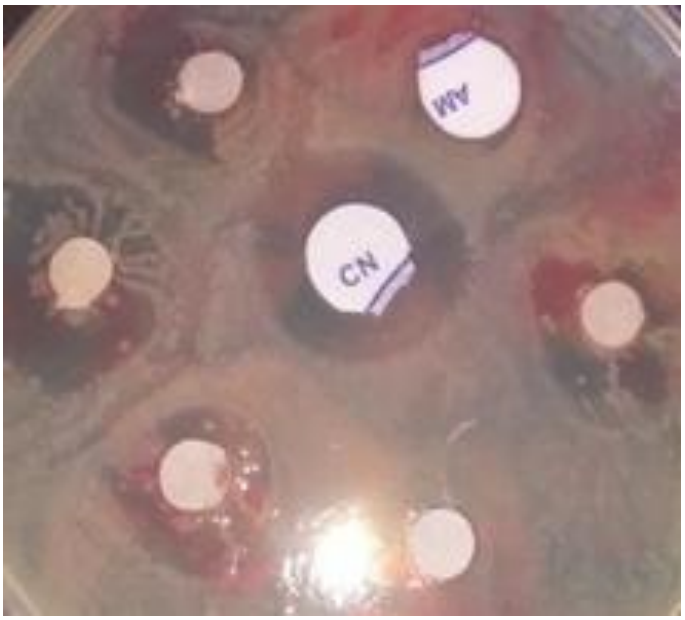

Fig. 1. Disc diffusion assay of E. coracana extract against *E. coli*

At 50mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control), and with the PBS (negative control) as described by Rupesh *et al*. in [14]. At 0h, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hs the vessels were seen to increase in number and dilate, the same was done after 48hs and 72hs. The average percentage of angiogenesis was 100% for both 50mg extract and extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative

control) (See Table 3). Two of the embryos were lost after 48hs of exposure to the extract.

At 100mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control), and with the PBS (negative control) as described by Rupesh *et al*. in [14]. At 0h, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hs the vessels were seen to increase in number and dilate, the same was done after 48hs and 72hs. The average percentage of angiogenesis was 75% for 100mg extract and 100% for extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative control). There's a little different to the information on the work done by Wang *et al*. in 2004, in that, at 50mg we got a100% dilation and newly formed vessels then at increased dosing, we got a reduction. This is likely due to the loss of an embryo or a feedback system from the protest due to an increase in the dose [13] as presented in Table 3.

At 150mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control), and with the PBS (negative control) as described by Rupesh *et al*. in[14]. At 0h, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hs the vessels were seen to increase in number and dilate, the same was done after 48hs and 72hs. The average percentage of angiogenesis was 91.67% for 150mg extract and 123.077% for extract plus 10µL bFGF while there was no dilation or new blood vessels on egg treated with PBS (negative control). This was compared to the information on the work done by Wang *et al*. in 2004. Good a thing, there was no loss of an embryo after exposure to the extract. Here, there was a potentiation of extract function by the bFGF (Carolyn *et al*., 2009). This is as shown in Table 3.

At 200mg of extract, the five (5) eggs were treated with the extract alone, the extract plus the bFGF, the bFGF alone (positive control), and with the PBS (negative control) as described by Rupesh *et al*. in [14]. At 0h, the prominent blood vessels were located and the extract impregnated methylcellulose discs were dropped. The disc was also treated with 10µL bFGF. At 24hs the vessels were seen to increase in number and dilate, the same was done after 48hs and 72hs. The average percentage of angiogenesis was 100% for 200mg extract and 105.26% extract plus 10µL bFGF, while there was no dilation or new blood vessels on egg treated with PBS (negative control). The glaring presence of the bFGF indicates the potentiated effect of the extract [13]. This is as shown in Table 3.

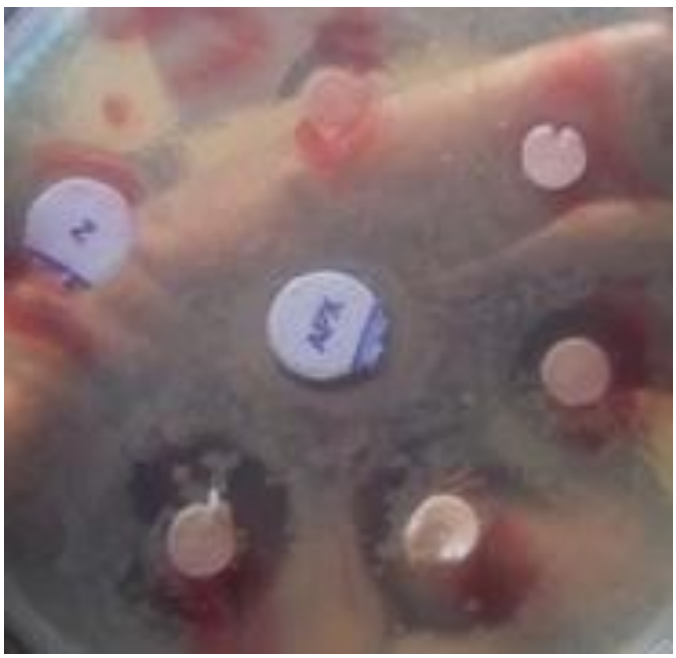

Fig. 2. Disc diffusion assay of *E. corocana* seed extract against *S. aureus*

Table 3. Percentage Angiogenesis of the *E. coracana* Ethanolic crude extract in the CAM assay

Conc.	Time	Initial Blood Vessels		New and Dilated Blood Vessels		Percentage Angiogenesis (%)	
		Ext	Ext+bFGF	Extract	Ext.+bFGF	Extract	Ext.+bFGF
	Neg. Con	0	0	0	0	0	0
50mg	0h	5	5	5	5	22.7273	29.4118
	24h	10	5	15	10	45.4545	58.8235
	48h	15	10	22	15	68.1818	88.2353
	72h	22	15	22	17	100.00	100.00
100mg	0h	1	3	1	3	12.5000	21.4286
	24h	7	8	8	8	87.5000	57.1429
	48h	8	8	8	8	100.000	57.1429
	72h	8	14	6	14	75.0000	100.000
150mg	0h	2	1	2	1	16.6667	6.25000
	24h	7	6	7	6	58.3333	37.5000
	48h	12	12	12	12	100.000	75.0000
	72h	12	16	11	13	91.6667	123.077
200mg	0h	3	3	3	3	13.6364	15.0000
	24h	10	8	10	8	45.4545	40.0000
	48h	22	20	22	20	100.000	100.000
	72h	22	20	22	19	100.000	105.2632
B FGF	0h	2		2		28.5714	
alone	24h	7		7		100.00	
	48h	7		7		100.00	
	72h	7		7		100.00	

Key: Ext = crude extract; Conc.= concentration of extract; Neg.con= Negative control

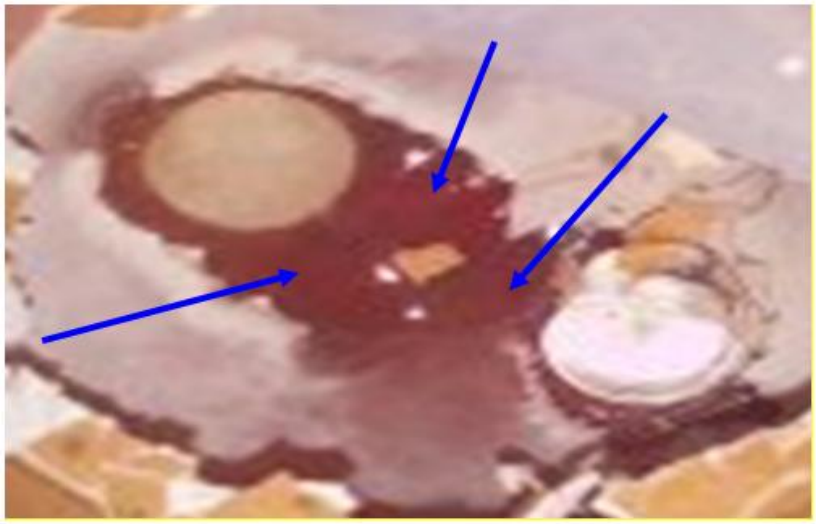

Fig. 3. A representative of CAM assay (200mg extract + bFBGF) after 48 hs of incubation, showing increase angiogenesis (indicated by blue arrows)

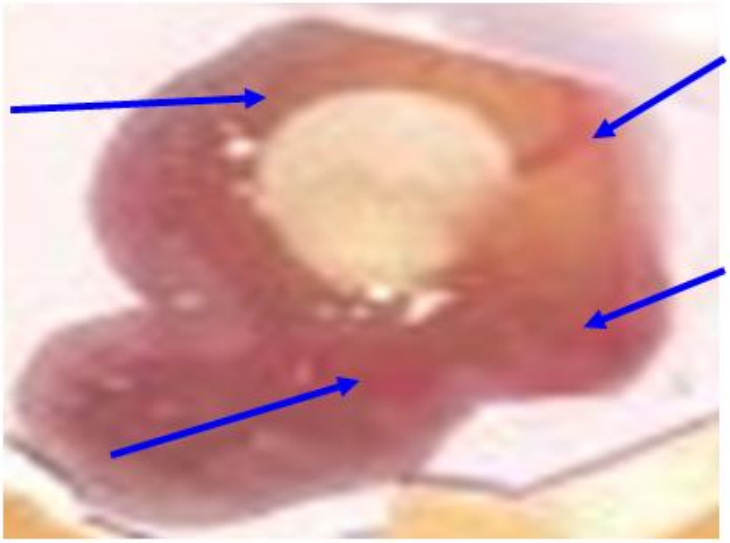

Fig. 4. A representative of CAM assay (200mg extract) after 48 hs of incubation, showing increase angiogenesis (indicated by blue arrows)

All results for the bFGF alone (positive control) are as shown in Table 3. 28.6% at 0h, while 100% at 24hs, 48hs, and 72hs formed the percentage angiogenesis on the blood vessels supplying the embryo.

3.4 Amino Acid Profile

An amino acid analysis was also key in looking at the usefulness of proteins in the wound healing cascade. The role of amino acids in wound healing is well established [17]. The synthesis, breakdown, and resynthesis of collagen a critical factors in the re-establishment of tissue integrity for wound healing. Many amino acids are very key in collagen synthesis; however, glycine is the most important followed by proline and hydroxyproline [17].

In this study, Glycine and Proline were all present in a significant amount. Thus, their presence can signify possible wound healing properties. The presence of tryptophan in minimal quantity reported here corroborated another study by Fernandez et al., [18] which also reported tryptophan in E. coracana seeds in low quantity.

Table 4. Amino acid content of the three fractions and the Ethanolic extract of *E. coracana* using PTH method

	Hexane fraction	Methanol Fraction	Aqueous Fraction	Ethanolic Crude Extract
Amino Acid	**Concentration: g/100g protein**	**Concentration: g/100g protein**	**Concentration: g/100g protein**	**Concentration: g/100g protein**
Leucine	7.20	4.55	4.00	3.85
Lysine	5.04	2.41	2.70	1.24
Isoleucine	3.30	1.60	1.20	0.95
Phenylalanine	3.55	2.66	1.95	1.06
Norleucine	Internal std	Internal standard	Internal standard	Internal standard
Tryptophan	0.84	0.60	0.52	0.44
Valine	3.60	2.22	1.70	0.90
Methionine	1.23	1.04	0.96	0.75
Proline	3.35	2.23	1.83	2.03
Arginine	6.20	4.30	3.01	2.60
Tyrosine	3.44	3.10	2.06	1.03
Histidine	2.20	1.21	0.86	1.08
Cystine	0.85	0.54	0.36	0.54
Alanine	4.02	2.60	1.82	2.05
Glutamic acid	10.60	5.22	3.63	4.54
Glycine	2.32	3.00	2.10	1.23
Threonine	3.20	1.50	1.05	0.94
Serine	3.35	2.65	2.02	1.51
Aspartic acid	5.98	4.40	3.16	3.10

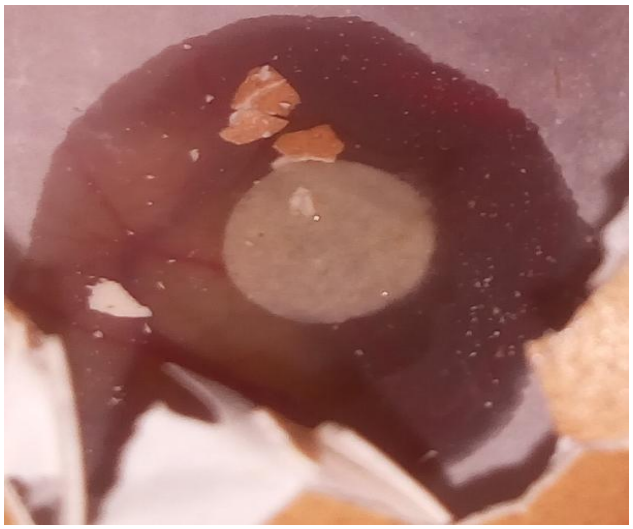

Fig. 5. A representative of CAM assay negative control (PBS only) after 48 hs of incubation, showing no increase in angiogenesis

4. CONCLUSION

The angiogenesis by fibroblast mediated activity yielded good percentages using the chorio-allantoin membrane assay indicates that the seed flour extract of *E. coracana* has good wound healing properties. The antimicrobial susceptibility of the seed extract of *E. coracana* showed clear zones of inhibitions against *E. coli, S. aureus, C. tetani* but none against *P. aeruginosa* which signifies its possible importance in wound healing. Also, the amino acid content of the extract might be responsible for the observed wound healing property.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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