The Journal of Anatomical Sciences
Email: journalofanatomicalsciences@gmail.com

J. Anat Sci 15(2)

Submitted June 7th, 2024
Accepted July 18th, 2024
Published September 30th, 2024

Costus afer Stem Juice Extract Enhances the Expression of HAS3 during the Wound Healing Process

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ABSTRACT

Among its various applications, the stem juice of *Costus afer* has gained attention in the scientific community for its potential in tissue repair and regeneration. This study was carried out to assess the expression of hyaluronic acid synthase 3 (HAS3) in wounds treated with *C. afer* stem juice extracts. Ninety-six adult Wistar rats were used for the study. The rats were equally divided into four groups: A, B, C, and D for each procedure. Each group was subdivided equally into four observational groups for the investigation of the healing process through 24 hours, 72 hours, 120 hours, and complete healing. The rats were wounded on their dorsum under ketamine hydrochloride anesthesia. The rats were treated only on the first day according to their groups. Group A wounds were left without treatment, group; B were treated with honey gel; group C were treated with heat-dried extract of *C. afer* stem juice; and group D was treated with freeze-dried extract of *C. afer* stem juice. The result of the study showed that both extracts showed similar patterns of HAS3 expression until the late stages of wound healing, during which expression of HAS3 in group D was decreased, while it was increased in group C. However, group D wounds showed the highest expression of HAS3 throughout the period of observation. In conclusion, the stem juice extracts of *C. afer* modulated the expression of HAS3 during wound healing, with higher expression levels from treatment with the freeze-dried extract.

Keywords: Wound Healing, HAS3, *Costus afer*

INTRODUCTION

Costus afer is a tropical plant that is well-known for its medicinal properties. In Africa, its stem juice is applied in the treatment of several ailments including malaria, rheumatism, hemorrhoids, and jaundice. Scientific studies have shown that its stem juice possesses antimicrobial, antioxidative, anti-inflammatory, and antinociceptive properties. These properties have been attributed to the presence of various biomolecules such as alkaloids, phenols, lignans, triterpenes, glycosides, saponins, and flavonoids [1–5]. Some of these biomolecules such as alkaloids, triterpenes and flavonoids have been found to influence cellular activities including, proliferation, migration, angiogenesis and collagen deposition [3,5–7].

A study by Udoh and colleagues [8] showed that an aqueous extract of *Costus afer* stem juice significantly improved wound healing giving a better wound healing outcome compared to wounds that were untreated. The study reported that the wounds treated with an aqueous extract of *Costus afer* stem juice showed a moist wound surface through most of the healing process despite being open. The researchers reported that the moist wound surface was observed both in wounds treated with aqueous extract of *C. afer* for seven days and those treated only on the first day. This implied that the ability of *C. afer* stem juice to induce and sustain moisture on the wound surface is due to its effect at the wound site during the early phase of wound healing. This effect could be through enhancing the expression of intrinsic molecules that promote moisture availability at the wound site, such as hyaluronic acid.

Hyaluronic acid (HA) is one of the chief components of the extracellular matrix. It is a linear non-sulfated glycosaminoglycan (GAG) composed of repeating units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA). In contrast to other GAG types, HA molecules do not undergo any form of

modification like sulphation or epimerization to produce isomers [9]. The only variables in relation to the systemic hyaluronan are the size of the molecule, determined by the length of the polymer, and the concentration of HA as well as its distribution across tissues [9,10]. Furthermore, the vast number of HA-binding proteins, called hyaladherins, contributes to the richness and robustness of HA interactions within cells and tissues. This hyaladherins show differentiated tissue expression, cellular localization, as well as binding specificity and affinity [10,11].

The HA molecules have the ability to bind water up to 1000 times its mass, making it one of the most hygroscopic molecules found in nature. They have a net negative charge that traps and impedes water molecules, creating a framework for cellular migration, differentiation and proliferation in the extracellular matrix, while maintaining the biochemical and structural integrity of tissues [9,11,12]. They play vital roles in the sequestration of reactive oxygen species, extravascular distribution of plasma proteins, and water-electrolyte homeostasis of the extracellular space [9,13]. Apparently, HA content determines the state of tissue hydration. It significantly contributes to the hygroscopic nature of other ECM molecules by organizing proteoglycans and other binding proteins to create highly hydrated and charged domains [12].

The biological effects of HA are directly dependent on its molecular weight. Hyaluronic acids with molecular weights from 0.4 to 4.0 kDa act as an inducer of heat shock proteins, and have a non-apoptotic property. HA polysaccharides with a molecular weight equal to 6–20 kDa possess immunostimulatory, angiogenic, and phlogotic activities. Hyaluronic acid with a molecular weight of 20–200 kDa takes part in biological processes such as embryonic development, wound healing and ovulation [12,14]. By contrast, high molecular weight hyaluronic acid (>500 kDa) polymers function as space fillers, antioxidants, and suppress angiogenesis, immuno-response and inflammation. They also inhibit cellular differentiation through intercellular interactions or ligand-receptor binding [9,15,16]. Thus, normal tissues are rich in high molecular weight HA (HMW-HA), which maintains homeostasis and promotes tissue stability. On the other hand, injured or diseased tissues are rich in low molecular weight HA (LMW-HA), which signals tissue damage and induce inflammation as well as healing process [11,14].

During wound healing, prolonged expression of LMW-HA contribute to sustained inflammation, immune-stimulation, angiogenesis and scar formation [17–19]. Usually, LMW-HA activates a negative feedback mechanism that promotes their phagocytosis by macrophages removing them from the wound site [17]. This process is critical for the resolution of the inflammatory process during wound healing. In contrast to LMW-HA, HMW-HA reduce scarring by inhibiting inflammation, oxidation, and angiogenesis [18]. HMW-HA enhances the availability of pro-healing cytokines and growth factors at wound site, and promotes the deposition and organization of fibrous proteins like collagen and elastin in the ECM [20,21]. According to David-raoudi *et al.*, [22], HA deposition at wound site rapidly decreases during the mid-stages of wound healing, with the appearance of proliferating fibroblasts that facilitate chondroitin sulphate and collagen deposition. This sudden decrease in HA content of adult wound has been attributed to high levels of hyaluronidases in the wound fluid [23,24]. It may also be due to the activity of hyaluronic acid synthases at the wound site [25,26].

Hyaluronic acid synthase 3 (HAS3) is one of the three isoforms of hyaluronic acid synthase, a group of enzymes that catalyzes the polymerization of UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc) into hyaluronic acid (HA) chains [27]. HAS3 is primarily expressed in connective tissues, such as the skin. It is a multi-domain protein consisting of a cytoplasmic domain, a transmembrane domain, and a catalytic domain. The cytoplasmic domain contains sites for substrate binding and regulation, while the transmembrane domain anchors the enzyme to the plasma membrane. The catalytic domain, located extracellularly, harbors the active site responsible for HA synthesis [25,27]. The enzymatic activity of HAS3 is regulated by substrate availability, enzyme localization, and protein-protein interactions [28].

HAS 3 typically synthesizes HA with molecular weight ranging from several hundred kilodaltons to several megadaltons. It generally has a preference for the synthesis of high molecular weight HA [25,29]. The size of the HA chains synthesized is influenced by the cellular microenvironment, regulatory mechanisms, and physiological context [25]. Among the HAS isoforms, HAS3 is a key regulator of HA production in response to tissue injury [30]. In skin wounds, it has been found that HAS3 expression is upregulated during the early stages of wound healing, promoting the synthesis of hyaluronic acid and facilitating the migration of fibroblasts and keratinocytes, which are essential for re-epithelialization and granulation tissue formation [29,31].

This study was carried out to investigate the expression of HAS3 at wound site as an indication to the synthesis of hyaluronic acid at the wound site. The premise for this study was based on the observation of moist wound surface by Udoh and colleagues [8].

MATERIALS AND METHODS

Fresh stems of *C. afer* were collected from bushes within Uyo Local Government Area. After removal of the foliage leaves, the stems were thoroughly washed and cut into pieces to be crushed in an electric blender. The juice obtained from the blender was filtered with a chess cloth. Equal volumes of the juice were concentrated by freeze-drying using a freeze-dryer and by evaporation in beaker placed in water bath at 40^{o}C.

Analyses of Phytoconstituents of Extracts

A portion of the extracts was collected and analyzed for their proximate, phytochemicals and mineral constituents.

Phytochemical analysis

The qualitative and quantitative analyses of the phytochemicals in the extracts were carried out according to the procedures described by Ezeonu and Ejikeme, [32], as well as Abubakar and colleagues [33].

Ethical approval

The ethical approval for this study was obtained from the Faculty of Basic Medical Sciences Research and Ethical Committee. The study was given the ethical number: UU_FBMSREC_2024_010.

Experimental animals

Ninety-six adult female and male Wistar rats, weighing between 240-320g, obtained from the animal house of the Department of Pharmacology and Toxicology, University of Uyo were used for this study. The rats were housed individually in plastic cages and allowed to acclimatize for 14 days. They were fed with pelletized diet (Vital Feeds Growers, Green Cereals Nigeria Ltd.) and water *ad libitum*, and exposed to 12-hour light/dark natural lighting.

Experimental protocol

The rats were equally divided into 4 treatment groups (24 rats each) of A, B, C, and D for each treatment procedure as illustrated in Table 1. Each treatment group was subdivided into four observational groups of 6 rats each for the investigation of the healing process through time. For each treatment group, treatment was done only on the first day after which six rats were chosen at random after appropriate treatment for molecular and histological assessments on 24 hours, 72 hours, 120 hours, and on complete healing (Table 1). The wounds were considered to be completely healed when the wound surface was fully covered with epidermis without any visible underlying blood vessel.

Table 1: Experimental design

Group	Treatment	24 hr	72 hr	120 hr	Healed	Total
A	No treatment	6 rats	6 rats	6 rats	6 rats	24 rats
B	Medihoney gel	6 rats	6 rats	6 rats	6 rats	24 rats
C	Heat-dried extract	6 rats	6 rats	6 rats	6 rats	24 rats
D	Freeze-dried extract	6 rats	6 rats	6 rats	6 rats	24 rats
Total		**24 rats**	**24 rats**	**24 rats**	**24 rats**	**96 rats**

The health status of the rats were closely monitored by regular assessment of their weight and stress indicators as reported in Sotocinal and colleagues [34]. All experimental handling of animals were according to National Centre for the Replacement, Refinement and Reduction (3Rs) of Animals in Research [34], as well as internationally accepted standard guide for care and use of laboratory animals promulgated and adopted by the National Institute of Health (NIH) publication number 85(23), revised 1996 and related ethics and regulation. All animals were handled humanely with care. Throughout the experiment, all procedures were carried out with high degree of asepsis.

Wound creation

All surgical interventions were carried out under local anesthesia with lidocaine. A predetermined area of fur, 5 cm from the ear centrally, was shaved off the rat dorsum using scissors and razor blade. The shaved area was cleaned using savlon and methylated spirit. Within the shaved area, a circular marking of 1.5 cm in diameter was made using methylene blue to guide the excision process. The target area was excised with surgical blade, scissors and forceps to create a full thickness circular wound. Immediately after wounding, sterile gauze was used to apply pressure on the wound until haemostasis was attained. Thereafter, the wounded rats were treated according to their treatment groups.

Treatment of wounds

Four treatment groups, A, B, C, and D, were considered in the study as illustrated in Table 1. All groups were treated only on the first day of injury.

Group A served as control without treatment; group B was treated with honey gel (Medihoney), which served as standard treatment control; group C was treated with heat-dried extract of *C. afer* stem juice; and group D was treated with freeze-dried extract of *C. afer* stem juice. Medihoney gel was obtained commercially from pharmaceutical stores, while other treatment substances were developed during the study. A spatula was used to apply 2.5 mL of each treatment agent on the wound. Then the rats were left for observation according to the experimental protocol as illustrated in Table 1.

Microbiological assessment procedure

The microbial profile of the breeding environment was carried out to determine the presence or absence of certain microbes that could affect the healing process in case of future study replication. About 1 g of the bedding was placed in a sterile universal container, and 10 mL of tryptone soya broth was added. It was incubated at 37ºC for 24 hrs. The overnight broth was sub-cultured on nutrient agar, Macconkey agar, and Sabouraud dextrose agar. The plates were incubated at 37ºC for 24 hrs. The isolate bacteria were identified based on colony morphology, gram staining, reaction, and biochemical characteristics using established standardized methods according to Bergey's Manual of Determinative Bacteriology [35].

Examination and Description of Microscopic Specimens

Examination of slide (section) by the naked eye, then thorough examination of whole section using low power (x4) objective lens (Olympus microscope camera, Japan) so as to appreciate the main structural patterns and identification of normal tissue. Abnormal areas were subjected to further examination still under low power and medium power (x10) objective and doubtful cells or structures were further examined under higher (x40) objective). Normal tissues were identified, and the pathological changes observed in the microscopic examination were described accordingly based on histological structure of the tissues. Photomicrographs were taken with the aid of computerized digital camera (Amscope MU900, United States).

Protocol for immunohistochemical staining of skin tissues

Paraffin embedded tissues were micro-sections (4 μ), floated and mounted on charged glass slides. The slides were labeled, arranged in racks and placed in oven at 50-60ºC for 20-30 min to melt excess paraffin. The slide-mounted tissues were further deparaffinized and prepared for heat induced antigen retrieval (in citrate buffer solution (10 mM citric acid, pH 6.0). The staining was performed using the Thermo Scientific Pierce Peroxidase IHC Detection Kit (36000, Thermo Scientific, USA) with slight modification of the procedure. Endogenous peroxidase activity was quenched by incubating tissue for 30 min in Peroxidase Suppressor, washed three times in Wash Buffer. Blocking buffer was added to the slides and incubated for 30 min. Excess buffer was blotted from the tissue sections, before addition of primary antibodies at a dilution of 1:100, and left overnight in a humidified chamber at 4°C. Afterward, slides were washed two times for 3 minutes with Wash Buffer. The tissue sections were treated with Biotinylated Secondary Antibody and incubated for 30 min. The slides were washed three times for 3 min each with Wash Buffer, treated and incubated with the Avidin/Streptavidin-HRP for another 30 min, and again washed three times for 3 min each with Wash Buffer.

The tissues were incubated with Metal Enhanced DAB (3.3 diaminobenzidine) Substrate Working Solution for 5 minutes for desired staining to be achieved. The slides were rinsed with distilled water and drained. Adequate amount of Mayer's hematoxylin stain was dropped on the slide to cover the entire tissue surface and incubated for 1-2 min at room temperature. Drained off the hematoxylin and the slides were washed several times with distilled water. The slides were mounted with cover slips using DPX mountant.

Photomicrographs were taken with digital camera (Amscope MU900) attached to the microscope. The images were quantified for staining intensity using the open source Fiji (ImageJ) software.

RESULTS

Phytochemical analysis of *C. afer* stem juice extract

Both extracts of *C. afer* stem juice were found to contain alkaloids, triterpenes, saponins, glycosides, lignans, phenols, and flavonoids. Both contained high amounts of phenol, while saponins were found to be of lowest concentration in the freeze-dried extract, and triterpenes were lowest in the heat-dried extract (Table 2). Generally, the freeze-dried extract contained more concentrations of the biomolecules than the heat-dried extract.

Table 2: Percentage concentration of biomolecules in *C. afer* stem juice extract

S/N	Biomolecules	Freeze-dried Extract (%)	Heat-dried Extract (%)
1	Alkaloid	3.79	2.41
2	Triterpenes	1.64	1.23
3	Saponins	0.81	0.65
4	Glycosides	5.7	3.1
5	Tannins	4.81	3.03
6	Phenol	42.6	21.5
7	Flavonoids	11.82	10.7

Microbial profile of experimental environment

The classes of microbe identified and isolated from the beddings that the rats were bred on were *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*, *Candida spp* (Table 3). While these microbes were present in the environment of the rats, no clinical signs of infection such as purulent discharge, foul odour, and swelling, were observed in the wounds through the healing process.

Table 3: Microbes identified in experimental environment

S/N	Microbe	Identification
1	*Pseudomonas aeruginosa*	Present
2	Staphylococcus aureus	Present
3	*Escherichia coli*	Present
4	*Proteus mirabilis*	Present
5	*Candida spp*	Present
6	Klebsiella pneumonia	Present

Expression of HAS3 in wound sites

Throughout the periods of observation, HAS3 expression was highest in group D wounds (Figure 1). It was lowest in group B and group C wounds at 24 hours and 120 hours, respectively. At 72 hours and on complete healing, group A wounds showed the lowest expression of HAS3.

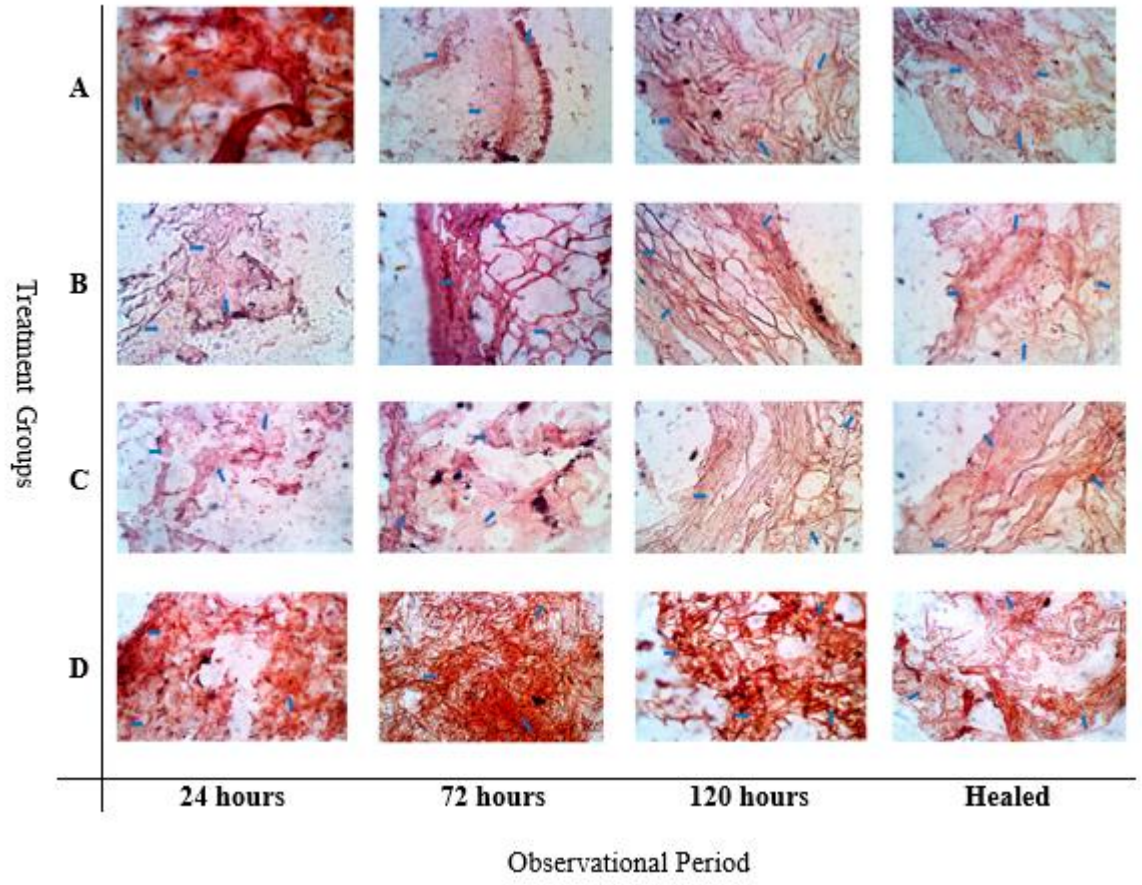

Figure 1: Immunohistochemistry micrograph of HAS3 expression (brown pigment, blue arrows) within each treatment groups across observational periods. At 24 hours, HAS3 expression was higher in groups A and D, but lower in groups B and C. At 72 hours, the expression of HAS3 in group A dropped to the lowest level among the groups, while it was increased in group B, with group D showing the highest level of expression among the groups. At 120 hr, the level of expression of HAS3 in groups B and C decreased, while that of group A increased slightly, with group D still showing the highest level of expression. In the healed tissues, the level of expression of HAS3 appeared to be similar across the treatment group, except for group D showing a slightly higher level of expression. (x100)

Intensity quantification of HAS3 expression

Intensity quantification of the immunohistochemistry micrographs showed that Group D wounds treated with *C. afer* freeze-dried extract had the highest level of HAS3 expression at 24 hr, which increased slightly at 72 hr and gradually reduced through 120 hr to levels comparable to other treatment groups in the healed tissue (Figure 2). Although the level of expression of HAS3 at 72 hr, and 120 hr were lower in group C treated with *C. afer* freeze-dried extract, the pattern of expression was similar to that of group B treated with honey gel (Figure 2). Data analysis using ANOVA showed that at 24 hr the level of HAS3 expression in groups A and D were significantly different from those of groups B and C (Table 4). At 72 hr, there was no significant difference in the level of HAS3 expression between groups B and C, while the levels of expression in groups A and C were each significantly different from other groups (Table 4). At 120 hr, there was no significant difference in the level of HAS3 expression between groups A and B, while the levels of expression in groups C and D were each significantly different from other groups (Table 4). In the healed tissues, there was no significant difference in the level of HAS3 expression between groups A, B and C, while the levels of expression in groups D was significantly different from all other groups except for group B (Table 4).

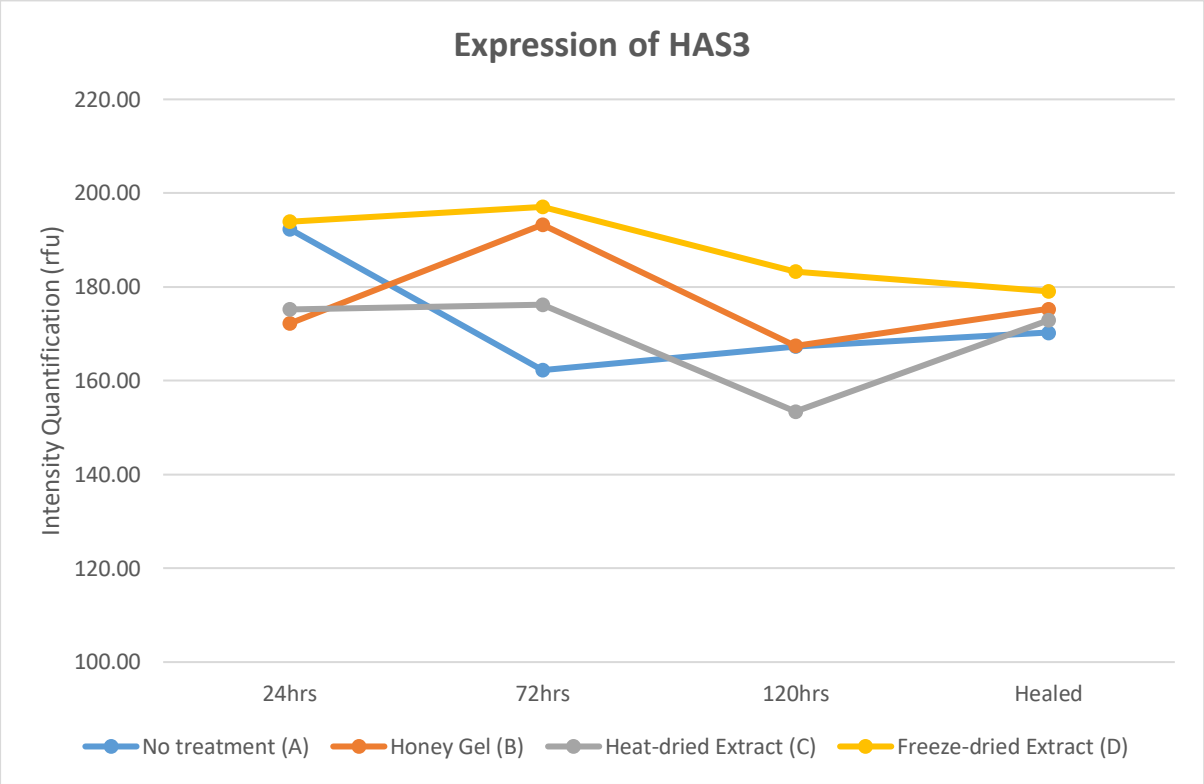

Figure 2: Pattern of expression of HAS3 across treatment groups.

Table 4: Intensity quantification of HAS3 expression at wound site.

Treatment Groups	24 hours	72 hours	120 hours	Healed
No Treatment (A)	192.36±0.28^{a}	162.26±0.44^{a}	167.29±0.89^{a}	170.24±0.89^{a}
Honey Gel (B)	172.23±0.99^{b}	193.28±0.59^{b}	167.43±1.42^{a}	175.31±0.51^{ab}
Heat-Dried Extract (C)	175.24±0.54^{b}	176.22±0.89^{c}	153.44±1.89^{b}	172.92±0.57^{a}
Freeze-Dried Extract (D)	193.94±0.91^{a}	197.07±0.61^{b}	183.31±1.95^{c}	179.07±1.05^{b}
p-value	0.00	0.00	0.00	0.01

a,b,c: values with different superscripts are significantly different at $p < 0.05$

DISCUSSION

The expression of HAS3 in the *C. afer* treatment groups showed similar pattern through 72 hours and 120 hours, although the expression levels were higher in the group treated with freeze-dried extract of *C. afer* stem juice. Clearly, the similarity in the expression pattern indicates their common origin from the same plant. The difference in their expression levels corresponds to the level of concentration of biomolecules in each extract, in which the freeze-dried extract had the highest concentrations of biomolecules. Particularly, the pattern of expression of HAS3 from the group treated with freeze-dried extract of *C. afer* stem juice corresponds to the report by Olczyk and colleagues [9], which showed that HA concentration is highest during granulation tissue formation, and gradually decreases in subsequent stages of healing reaching its basal concentration during the remodelling phase.

Although, direct evidence linking the biomolecules in *C. afer* to HA synthesis is lacking, their anti-inflammatory and antioxidant activities may point to their possible effect on HA synthesis and expression, as HA, particularly high molecular weight HA is primarily involved in inflammatory resolution and ROS scaffolding within tissues. Thus, the high expression of HAS3 in the group treated with freeze-dried extract of *C. afer* stem juice corresponds strongly with available literature, as almost all the biomolecules identified in the extract including alkaloids, triterpenes, glycosides, flavonoids, and

phenols have been shown to exhibit anti-inflammatory properties [4,36–39], which could be through the simulation of HMW-HA synthesis by HAS3. In addition, a number of polyphenolic compounds, tannins, triterpenes and alkaloids have been reported to exhibit strong antioxidant activities [2,3,40,41], which could also be related to their possible ability to stimulate HMW-HA synthesis.

Unlike the findings of Udoh and colleagues [8], moist wound environment was not observed in all the wounds, and for those that were observed, it did not last through most of the healing period. This could be due to the dry and windy weather of the harmattan season during which the study was carried out, as the experimental room was exposed to the natural weather condition. Notwithstanding, considering the expression levels of HAS3, it is possible that the downstream synthesis of HA is responsible for the maintenance of moisture in the open wounds treated with *C. afer* stem juice extracts. This is in agreement with the hygroscopic nature of HA, and its role in hydrating the ECM [9,13].

Furthermore, the ability of *C. afer* stem juice to enhance the expression of HAS3 during the wound healing process suggests its potential in modulating repair and regeneration properties of other tissues that are mostly dependent on the expression of hyaluronic acid. According to Valachová and Šoltés [13], HA mediated signalling is critical for most of the cellular responses during repair and regeneration, such as cell attachment, migration, and proliferation. Kavasi and colleagues [21] also reported that HA, particularly HMW-HA, play crucial roles in ameliorating inflammatory diseases by scavenging cytotoxic free radicals such as reactive oxygen species. Thus, by enhancing HAS3 expression, *C. afer* stem juice shows potential for its application in the treatment of inflammatory diseases. In addition, HAS3, as well as its product HA, has been found to enhance the production and availability of growth factors such as TGFβ-1, VEGF, and PDGF at wound sites, which are also involved in the repair and regenerative processes of other tissues [9]. This further indicates the potential role of *C. afer* stem juice in tissue repair and regeneration therapeutic applications.

Conclusion

This study showed that the extract of *C. afer* stem juice promotes the expression of HAS3, which could contribute to the maintenance of moist healing environment through the synthesis of hyaluronic acid.

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