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Analysis of the Efficacy of Topical Aqueous Creams Containing AzadirachtaIndica Leaf Extract for Healing Wounds

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Abstract

In context, wounds are a kind of health problem that may have serious monetary and social consequences for the person who sustains them and their loved ones. Azadirachtaindica leaf aqueous extract (AEAIL) has been shown to have wound healing capability. The AEAIL may be more useful as a wound therapy if it were developed into a topical aqueous cream.

Objectives: Using hydroxyproline (HXP) as a biochemical marker, the purpose of this work was to manufacture aqueous topical creams containing different concentrations of AEAIL as bioactive components, and then to assess their stability and wound healing activity in male Wistar rats. Using DMSO, cholesterol, and distilled water as controls, we tested the wound healing capabilities of creams containing 1.0, 1.5, 2.0, and 3.0% w/w AEAIL on male Wistar rats over the course of 14 days. The animals treated with the cream containing 1.5% w/w of AEAIL had the greatest tissue HXP level ($p > 0.05$), and all batches of cream were stable in terms of color, pH, viscosity, etc. Animals given DMSO, cholesterol, or distilled water had decreased tissue HXP levels compared to those given test creams (p 0.05). Their HXP levels were somewhat lower than those of the control creams, but the difference was statistically significant (p 0.05). The wound-healing properties of an aqueous cream containing an extract of Azadirachtaindica leaves were shown to be stable. As a result, this novel formulation may be employed to heal wounds to the human body.

Key words: Wound healing; Aqueous cream; *Azadirachtaindica*leaves; Bioactive ingredient; Hydroxyproline;Wistar rats

Introduction

Injuries to the skin or other hard tissues of the body are known as wounds [1]. It weakens the victim's social network and their ability to make ends meet [2]. Damage to tissue can be caused by a variety of factors, including physical contact, chemicals, heat, bacteria, and the immune system [3, 4]. Damage is typically classified according to its severity, recovery rate, underlying pathology, mortality risk, and impact on the victim's quality of life [5, 6]. An open wound is defined as a break in the skin caused by a cut, abrasion, or a puncture. A closed wound is recorded if a bruise results from a blunt force trauma. A burn is an injury caused by direct contact with an oxidizing or reducing agent, such as fire, heat, radiation, chemicals, electricity, or sunlight [3,4]. Recovering from an injury is a long and complex process that requires the injured part of the body to undergo a series of cellular and biochemical reactions that will lead to the restoration of the fundamental and functional constitution of the tissues as they were before the injury.

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There must be continuous cell-cell interface and cell-matrix interactions for the process to go through its many stages and processes, such as inflammation, wound contraction, re-epithelialization, tissue re-modelling, granulation tissue growth, and angiogenesis. When an injury occurs, the body goes through a series of stages of restoration until healing is complete; if any one of these stages fails, the body may not heal as expected, which could result in either a chronic wound like a venous ulcer or pathological damage like a keloid scar [7]. Stopping the bleeding is the first step in the wound healing process [8, 9]. Vascular constriction, platelet emigration, andShortly after arterial injury, coagulated fibrin forms to restore haemostasis and make way for an extracellular network where cells may migrate. This is how wound healing mediators bring inflammatory cells to the wound site, which is necessary for the next phase of inflammation [10]. This second phase, which involves haemostasis and clotting, begins a few hours after the injury and overlaps with the first. The accumulation of leukocytes and macrophages [9,11] is distinctly different from this stage. Injured areas are flooded with macrophages, which behave like platelets by releasing growth factors that promote the formation of new connective tissue or granular tissue [9]. Within three weeks after skin injury, macrophages assist resolve inflammation and spark tissue regeneration, allowing the transition from the inflammatory to the reparative phases of proliferation and remodeling. Granulation tissue creation, re-epithelialization of the wound surface, and contraction of the wound borders are signs of the third phase of wound healing, called proliferation [8,11]. Macrophages, fibroblasts, and immature collagen are all components of granulation tissue that are thought to stimulate its production. At the same time, new capillaries will form due to the stimulation of blood vessels. Collagen, a crucial component of the extracellular matrix, is synthesized by fibroblasts at the wound's surface. Reorganization of collagen fibers during the generation of new skin (stage four) may take a very long period [8,11]. Within three weeks of the damage, new skin could progress less than a fraction of its full strength, and it almost never reaches the strength of the original skin [9].

In the past, several plant extracts have been used to treat various types of wounds. Wound healing is aided by the use of plant extracts that promote blood coagulation, kill bacteria, and speed up the healing process. Clarifying and researching phytoconstituents from plants for their usefulness in wound treatment is necessary [1].

Therapeutic application of Azadirachtaindica, sometimes known as neem, has been developed for the treatment of wounds, incisions, and other skin problems. The antioxidant effect of its flavonoids is maintained, protecting cells and tissues from harm caused by free radicals. Tannins in it promote recovery from injuries [12-14]. Many different phytoconstituents have been found in A. indica [15]. It has been shown in the scientific literature that A. indica leaf extracts (both ethanolic and methanolic) have wound healing capabilities [16, 17]. Using GC-FID methods, the phytoconstituents of AEAIL have been quantified [18]. It has also been shown that AEAIL possesses phytomedicinal and nutraceutical advantages [19]. The optimal effective concentration for wound healing in male Wistar rats was established to be 1.5% w/v of its crude extract, according to a recent study [18] that used hydroxyproline (HXP) as a biochemical marker to evaluate the level of collagen formation in wound healing. This research aimed to create an aqueous cream containing the AEAIL and test its stability and wound healing potentials in male Wistar rats. The pace of wound contraction, but more importantly the amount of tissue HXP observable in the healed wound area in male Wistar rats, will be used to evaluate the level of wound healing activity of the AEAIL added into the topical creams. The enzyme prolyl hydroxylase, which needs vitamin C as a co-factor, converts the amino acid proline into the non-essential amino acid derivative HXP during post-translational protein modification. The protein collagen relies on HXP for the integrity of its three-stranded structure [20].

Materials and methodsMaterials

The following materials were used for the studies as procured and include hydroxyproline assay kit (Elabscience,China), dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA), cholesterol (Molychem, India), emulsifying wax,liquid paraffinandsoftparaffin(Kerax, UK).

Methods

Collectionandextractionofthesampleof*Azadirachtaindica*leaves

Fresh neem leaves used had been identified by a Taxonomist and deposited in the University of Port Harcourtherbarium (voucher no. EH/P/070) as reported by Ugoeze*et al* [18,19]. The method of sample collection andprocessingasalso reportedbyUgoeze*et al*[18,19] wasadopted.

Formulationofaqueouscreamcontaining aqueousextractof *A.indica*leaves

Theaqueoustopicalcreamscontainingvariousconcentra

tionsoftheaqueousextractof*Azadirachtaindica* 1.
leaves(AEAIL)werepreparedusingtheformulainTable

Table1: Formula forthepreparationoftopicalcreamscontainingdifferentconcentrations ofAEAIL

Ingredients	**Batches/Composition(%w/w)**			
	A	B	C	D
AEAIL	1.0	1.5	2.0	3.0
DMSO	5.0	5.0	5.0	5.0
Cholesterol	10.0	10.0	10.0	10.0
Glycerol	5.0	5.0	5.0	5.0
Emulsifying ointment	20.0	20.0	20.0	20.0
Water,q.s.	100.00	100.00	100.00	100.00

Evaluationofthestabilityofcreams

The color and texture of the cooled creams were judged only by sight. Homogeneity was measured by creaming and phase separation, and consistency was determined by rubbing a sample between the fore and first fingers. Each cream batch was measured out and kept in a plastic container with a wide opening at -5 degrees Celsius, room temperature, and 40 degrees Celsius. Changes in the samples' color, look, consistency, and homogeneity were tracked everyday for 14 days.

Using a pH meter (Ultrameter II, 6PFC E; Myron L, UK), we measured the creams' pH in each batch. For 14 days, their pH was tracked while they were kept at room temperature [21]. The spreadability of the creams was also measured by putting a known amount of each cream between two slides and placing a 100.0 g weight on it for 10 seconds, then measuring the distance moved by the slides.

Viscosity

A Brookfield viscometer (Brookfield DV2TLVTJO, USA) with spindle no. 62 was used to measure the creams' viscosities at room temperature every other day for 14 days. Wound excision procedures in male Wistar rats for evaluating the creams' wound-healing effects

Thirty-five adult male Wistar rats weighing 200-250g were obtained from the animal house at the Faculty of Pharmaceutical Sciences, University of Port Harcourt and housed in individual cages for two weeks to acclimate to their new environment, during which time they were provided with free access to standard feed and water and maintained in standard conditions, including temperature (25-29), relative humidity (55-66%), and natural dark/light cycle. Each rat received an intramuscular injection of ketamine (50 mg/kg) to induce anesthesia. The dorsal region was shaved, cleaned, and a 1.5 cm x 1 cm full-thickness open incision was excised [22]. The care of the animals at the University of Port Harcourt was performed in accordance with all applicable ethical standards. The study followed the guidelines for the ethical treatment and use of animals in scientific research [23]. The University of Port Harcourt's Research Ethics Committee reviewed and approved all animal experimentation protocols (APPROVAL REFERENCE NO. UPH/CEREMAD/REC/MM71/043).

Animal model assessment of aqueous creams' wound-healing efficacy

Thirty-five male Wistar rats, all adults, were randomly assigned to one of seven groups (n = 5). Creams containing 1, 1.5, 2, and 3 percent w/w AEAIL were used on Groups 1 through 4, whereas DMSO, cholesterol (2% w/v), and distilled water were used on Groups 5 through 7. Researchers used DMSO and cholesterol to determine how wound healing is affected by these factors apart from the AEAIL. Wound contraction was measured before each treatment session, and the wounds were cleaned and treated every day until they healed. Using equation 1 below, [24] we were able to determine the proportion of wound contraction.

$$Percentageofwoundcontraction = \frac{Initialwoundsize - Specificdaywoundsize}{Initialwoundsize} x100 \quad (1)$$

Determinationoftissuehydroxyproline

Atthecompleteclosureofmostofthewounds,therat sweresacrificed.Tissuebioassaywasconducteddus ing100mgofthetissuescollectedfromthesiteofthe healedwoundoftherespectiverats,addedto1mlof6 M

hydrochloric acid, boiled for 6 h and cooled. The pH was adjusted to 6.8 while the volume was made up to 10 mlusing distilledwater. Each sample was centrifuged and 1ml of its supernatant was usedfor the assay of HXPlevel using the HXP kits (Elabscience, China) and conducting the experiment based on the protocols outlined inthe manufacturer's manual which is in line with the methods described by Bergman and Loxley [25] after theprinciplethattheoxidationproductproducedb yHXPundertheactionofanoxidantreactswithdi methylaminobenzaldehyde(DMAB;Ehrlich'sre agent)showingapurplishredcolour.TheHXPwas calculated by measuring the absorbance at 550 *n*m using a UV-VIS spectrophotometer (Jenway 6405, UK). Thevalueswere reportedasμg/gdryweightoftissue.

2.2.6Statisticalanalysis

The figures were presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) wasperformed followed by Fisher's Least Significant Difference (LSD) post hoc test to determine the level ofsignificance.

ResultsanddiscussionEvaluationoftheaqueousc reams

A homogeneous greenish smooth and consistently stable creams were obtained. It is essential to consider asuitable pharmaceutical formulation that enhances the optimal delivery of the active constituent leading to theefficacy of the preparation. Such formulations should be considered as suitable for the management of openwounds, which among other features, should be easily spread with emollient characteristics. The AEAIL wasformulated as a cream employing the principles of oil-in-water emulsions [26]. The formula and the constituentsemployedinthepreparationofthebat chesofcreamswereshownin Table1.Theincorporationoftheemulsifyingoint mentenhancesthestabilityoftheoil-in-watercreamformed,establishingthehydrophilicc omponent. Its presence with water and glycerol provide effective emollient and moisturizing effect whichenablesthereductionofdrynessandirritatio nofthedamagedskinasanocclusivebarrierisform edontheskinto inhibit the escape of moisture from the skin. Hydration of the *stratum corneum*permits the opening up of intraand intercellular channels for ease of penetration of active ingredients into the cells and injured tissues. Aneffective topical dermatologic formulation is also certain since water, glycerol and the emulsifying ointmentforms the hydrophilic component of the formulation which serves as a vehicle to solubilize and disperse theextract in the non-aqueous phase of the cream and also supports the mixability and penetration of the extract inthe hydrophobic component of the skin [27]. The DMSO and cholesterol contained in the formulation act asamphipathic surfactants to provide further stability to the formulation especially imparting hydrophobic andhydrophilicpropertiestothecreamandenhanc

ethestabilityoftheplantextractactingastheactive constituentto retain its activity in the environment of the various adjuvants. In addition to its action as an amphipathicsurfactant, DMSO also acts as a penetration enhancer [28,29]which is expected to improve thepenetration ofthe plant extract into the tissue to boost activity. Additional aspects of the oil-in-water based cream take accountof easy washability and high skin pore occlusion efficiency. Mostly, occlusion of wounds has been recognized toexpressivelydecreaseinflammationwhichamo untstodeclineinpainsandscaring[30].Decreaseo fpainandinflammation as well as speeding up of wound healing has been improved with moist healing environment andsuchconditionshavebeenattainedusingoil-in-watercreamswhichtallies withanocclusiveformulation[31,32].

The stability of the cream is critical to its effectiveness and safety. The appearance and consistency of theformulations were used to assess the stability of the creams as apparent instability may appear as a change incolour and/or consistency. Considering these features, there were no indications of coalescence, change in colouror inconsistency in the creams in the various stress situations they were exposed to. Spoilage or instability insome creams could occur as alterations in pH which gives rise to unwelcome experiences like skin irritation [33].The influence of storage time on the pH of the creams is shown in Figure 1. A statistically significant variation inthe pH of the creams were recorded ($p < 0.05$), with pH decreasing as the concentration of the AEAIL increasedfrom 1.0 – 2.0% w/w ($p < 0.05$) with no statistical difference in the pH of the creams containing 2.0 and 3.0 %w/woftheAEAIL($p>0.05$)(Figure2).Themean pHof5.760±0.001,5.750±0.005,5.740±0.001an d5.730±0.001wasrecordedforthebatchesofcrea mscontaining1.0,1.5,2.0and3.0%w/wofAEAIL respectively as at the 14th day of storage. These values, however, are very close to the pH of the skin of 5.7 for anaverage adult [34]. A stable pH of the respective batches of creams was recorded (Figure 1) as the storageprogressed to the14thdaysignifyingthestabilityofthecreamsco ntainingdifferentconcentrationofextract

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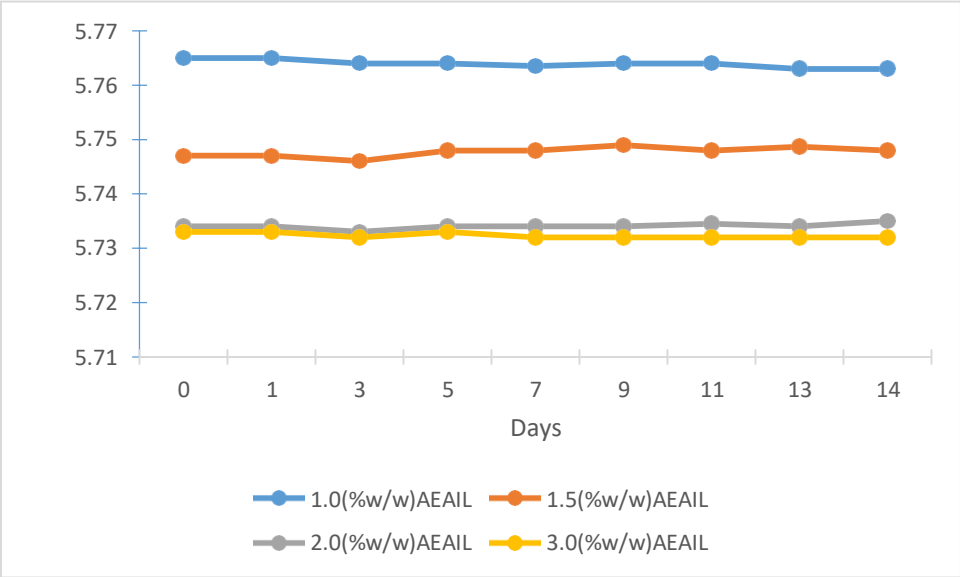

Figure1:pHofcreamsfollowingseveraldaysofassessment

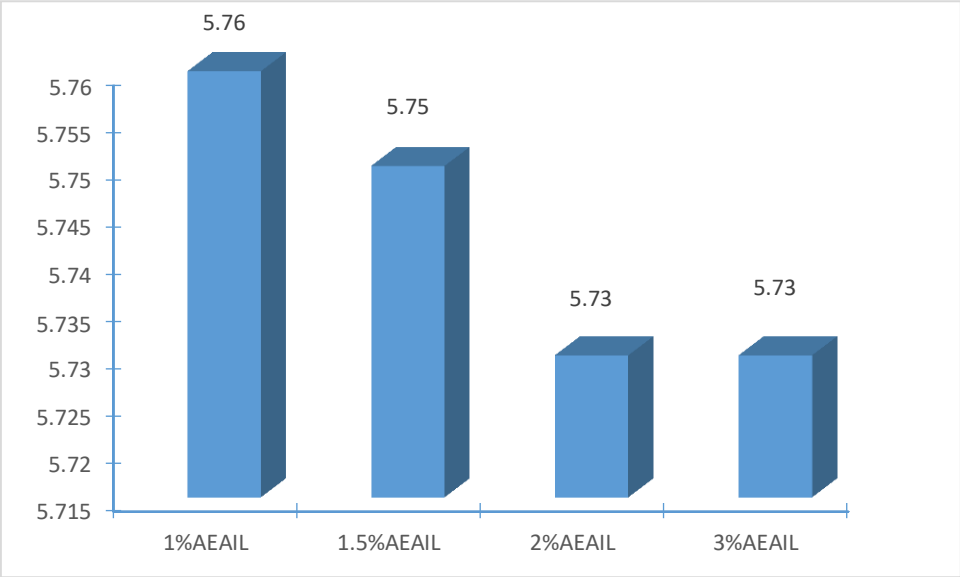

Figure2: MeanpHofcreamscontainingdifferentconcentrationsoftheAEAIL

The viscosities of the respective batches of creams are presented in Figure 3, showing variations in the viscositiesof the batches of creams containing different concentrations of the AEAIL ($p < 0.05$), though there was noconsistentpatternofvariation oftheirviscosities.Theviscositiesofthe batchesofcreamswerestable.

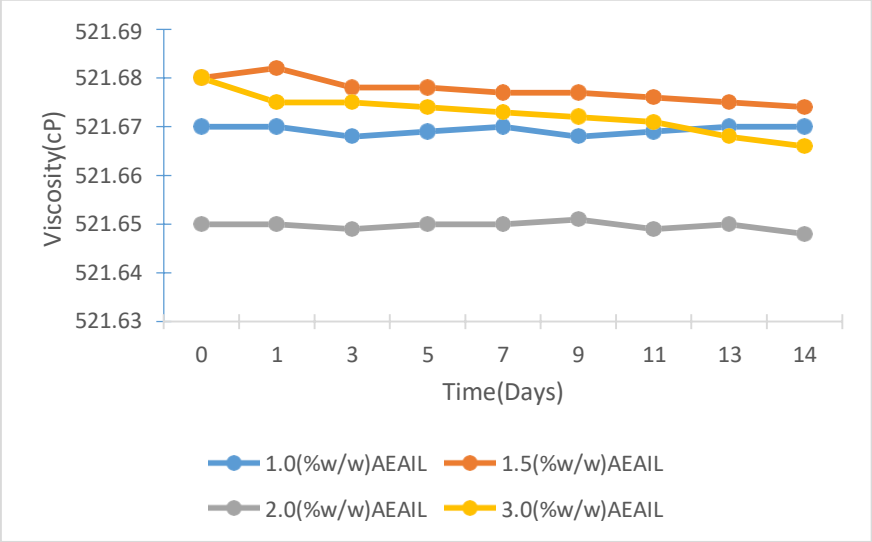

Figure3: Viscosityofcreamsfollowingseveraldaysofmeasurement

Evaluationofthewoundhealingeffectsofthe creams

Figure4showsthepatternofcontractionofwoundsfollowingthetreatmentwiththecreamscontaining1.0,1.5, 2.0 and3.0%w/wofAEAILandDMSO,cholesterolanddistilledwaterservingascontrols.Therewasa

continuous contraction of the various wounds with complete wound closure achieved in the 20, 19, 12, 13 and20th day of treatment for the creams containing 1.0 and 1.5% w/w AEAIL, DMSO, cholesterol and distilled waterrespectively. As at the 21st day of treatment, those treated with the creams containing 2.0 and 3.0% w/w ofAEAILshowed98.67and98.66%closure respectively.

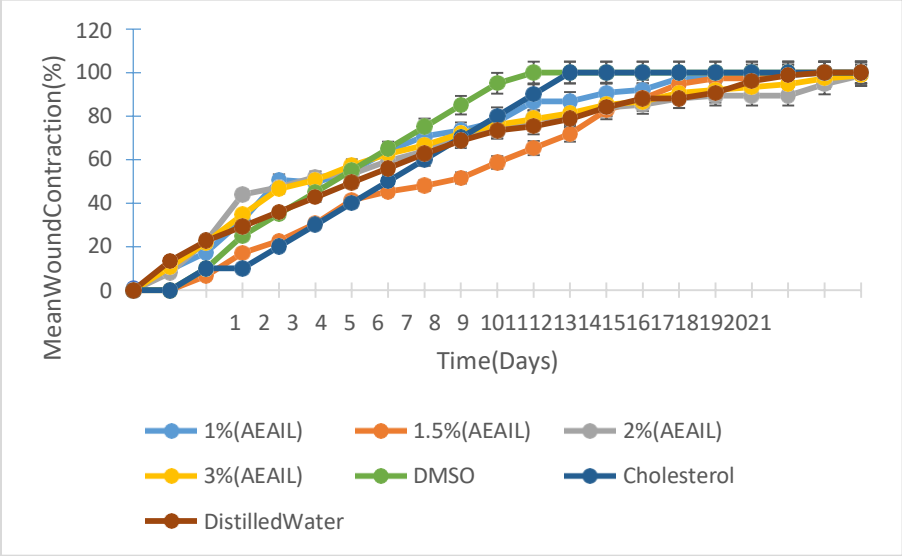

Figure4: Meanwoundcontractionfollowingthetreatment withcreamscontainingvariousconcentrationsoftheAEAIL

Resultsoftissueassayofhydroxyproline

The results of the tissue HXP assay is presented in Table 2 showing the mean tissue HXP levels obtained due tothe treatment of various wounds using the creams containing 1.0, 1.5, 2.0 and 3.0% w/w AEAIL and DMSO,cholesterol and distilled water as controls. The results showed that the highest tissue HXP level was obtainedfrom the group of animals treated with the cream containing 1.5% w/w of the AEAIL, though there was nostatistical difference in the mean tissue HXP levels of this and those obtained from the groups treated with thecreamscontaining1.0,2.0and3.0%w/wofAEAIL($p>0.05$),but,therewasasignificantdifferenceinthemean tissue HXP levels for the test creams and the entire control groups ($p < 0.05$) with marked percentagedifferences in their HXP levels (Table 2). Detection of statistically significant elevated mean tissue HXP in themale Wistar rats treated with the various creams compared to the control groupswas an indication of theretentionofthewoundhealingactivityoftheAEAILinthepresenceoftheadjuvantsemployedintheformulationofthecreams.Thisshowsthatthemeantissue HXP levelsdetectedinthegroupstreated withthetest creams were due to their contents of the AEAIL. In an earlier study, our research team confirmed the woundhealing potential of crude AEAIL in male Wistar rats using HXP as a biochemical marker and established itsoptimal wound healing concentration as 1.5% w/v [18]. The results of the present study have further confirmedthataminimalconcentration

mechanism of wound healing of the AEAIL may be attributed to its various phytoconstituents [18] based on theiranti-oxidant,anti-inflammatoryproperties, etc.

Conclusion

AqueousextractofAzadirachtaindicaleavescouldthereforebeusefulasabioactiveconstituentinthedevelopmentofanaqueoustopical creamusefulinthe treatmentofbodyinjuries.

Table2: Differenceinthemeantissuehydroxyprolinelevelsoftreatedgroupscomparedtothecontrolgroups(DMSO,cholesterolanddistilledwater)

Sample	Tissue HXP(µg/g)	VariationofHXPoftestsamplesfromcontrols			Remark
		DMSO	Cholesterol	Dist.water	
1.0%AEAIL	1.5767±0.03	20.82%	24.98%	49.99%	< 0.05
1.5%AEAIL	1.6300±0.09	24.90%	29.20	55.06	< 0.05
2.0%AEAIL	1.4753±0.27	13.05%	16.94	40.34	< 0.05
3.0%AEAIL	1.5594±0.04	19.49%	23.61	48.34	< 0.05
DMSO	1.3050±0.02	-	-	-	-
Cholesterol	1.2616±0.03	-	-	-	-
Dist.Water	1.0512±0.02	-	-	-	-

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