PharmacologyOnLine

Archives • 2021 • vol.2 • 1040-1049

BURN HEALING POTENTIAL OF *Annona muricata* L. ON SECOND-DEGREE BURN WOUNDS IN RATS

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Abstract

The present study aimed to evaluate the effect of cataplasm of *Annona muricata* L. leaves on second degree burns induced in a rat model. 32 specimens *Rattus norvegicus* albino, which were randomly distributed in four groups of 8 specimens, including the healthy, control, experimental (*Annona muricata* L.) and standard (silver sulfadiazine) groups. The treatments were topical and for 15 days. An experimental model was used for the induction of second-degree burns, by means of thermal burn. The variation of the burned body area (mm^2) at the lateral and craniocaudal level was evaluated daily and measurements of the lesions were carried out to control the healing during 15 days, then the histopathological study was carried out. In addition, a phytochemical study was carried out. The results show a significant reduction in the body area burned in the experimental and standard groups compared to the control group ($p < 0.05$), which is reinforced by the histopathological findings such as a decrease in blood vessels, fibroblasts and the presence of macrophages in the standard group, as well as a reticular-like chorion, greater presence of collagen and scarce fibroblasts and scarce blood vessels in the experimental group (*A. muricata* L) indicative of intense repair. Likewise, an abundant presence of flavonoids, tannins, alkaloids among other secondary metabolites was found in the leaves of *A. muricata* L. *Annona muricata* L leaves, administered topically, in the form of a cataplasm, revealed a healing effect on second-degree burns induced in the experimental model studied. Further studies are required to elucidate its mechanism of action and specifically determine the active principles responsible.

Keywords: *Annona muricata* L.; Healing; Second degree burns; Silver sulfadiazine; Histology.

Introduction

One of the public health problems that causes high death rates, approximately 180,000 deaths annually, is burns. Burns are defined as an injury or trauma of physical or chemical origin on the skin or other organic tissue, inducing denaturation of tissue proteins (1,2).

In Peru, there are no specific data on the death rate on burns or the occurrence of burns in all age groups, however, if there are studies related to the child population, referring that the most affected are those children who belong to strata medium or low socioeconomic levels, estimating an incidence of 2.1% (3,4).

Burns are classified based on depth, severity and the area covered by the injury, according to degrees, first degree burns are superficial because they only affect the outer layer of the skin, second degree burns are partial thickness and affect the epidermis and dermis, third degree or full thickness burns affect the epidermis, dermis and subcutaneous tissue, and finally fourth degree burns involve muscles and bones thus affecting nerve endings (5,6)

The causative agents of burns can be of different nature, for example, the main ones are chemical and electrical sources, heat from liquid, solid materials or fire, radiation, cold and friction (7).

Therapeutic strategies for the management of burns are based on the causative agent and the degree or severity of the same, however, the first treatment arises in first aid care, where the injured area must be delimited and subsequent infections avoided since the affected person is at vital risk and first aid determines subsequent morbidity (3).

Among the pharmaceutical products used in the treatment of burns are those with antiseptic properties such as products for topical use based on sulfonamides, silver salts, as well as colloidal solutions. Within the pharmacological treatment, drugs for pain and inflammation and the use of moisturizing creams in whose formulation is urea or lactic acid also stand out (6,8).

The treatments available in Peru for the management of burns are surgical methods, one of the most effective for reducing hospital stay and possible infectious contaminations, and the insertion of autografts is also included in this method. However, in other countries alternative treatments are being implemented that provide similar efficacy, and within these some formulations based on plant species play a fundamental role (3).

Studies are currently being carried out to validate the use of various alternative therapies based on medicinal plants whose traditional uses focus on their healing and healing properties, in which various parts of the plant such as leaves, fruits, bark and roots are used; highlighting within them the species *Allium sativum*, *Aloe vera*, *Centella asiática*, *Hippophae rhamnoides*, *Pinus halepensis*, *Cynara humilisy*, *Salvia verbenaca* (9–11).

Annona muricata L. (*Annonaceae*), known as graviola, soursop, guanábana, which is a fruit plant is dispersed both in the wild, and cultivated in the Antilles, the South of Mexico, Peru, Brazil, and the Pacific Islands. Also, it is cultivated in South Florida, Southeast China to Australia and the low and hot lands of East and West Africa. *A. muricata* is a small and branched tree, with thick and evergreen leaves, shiny at the bottom, of wide distribution, in which more than 50 acetogenins with different biological activities have been found present in fruits, bark and leaves (12,13).

The traditional uses reported for this plant are for the treatment of gastritis, inflammation, kidney problems and cancer; it's use is reported by oral administration of the infused soursop leaves (14).

Based on traditional use and especially due to its anti-inflammatory effects, the objective of this study was to evaluate the effect of cataplasm of *Annona muricata* L. leaves on second-degree burns induced in a rat model.

Methods

Obtaining and conditions of plant

The leaves of *Annona muricata* L. (guanábana), were collected in the San José Bajo village of the Santiago de Cao district, Ascope Province, La Libertad Department, Peru. The identification of the plant was confirmed by the *Herbarium Truxillense* (HUT) with code (HUT- No. 58837).

Selection of specimens

32 specimens *Rattus norvegicus* var. albinus, adult males, 3 to 4 months old and weighing approximately 200 to 250g, were purchased from

the National Institute of Health and housed in individual cages in the Bioterium of the Faculty of Pharmacy and Biochemistry of the National University of Trujillo. The specimens were kept under standard environmental conditions, with 12/12-hour light/dark cycles, 70% relative humidity, room temperature between 22 and 24 ° C, and fed a balanced diet and water *ad libitum*.

Preparation of the vegetable sample

From the collected leaves the preparation of the cataplasm pharmaceutical form was made, for which 50 g of *Annona muricata* L. leaves were weighed, which were previously dried and proceeded to crush them in a mortar, then 1 mL of water at 37 ° C, which made it possible to obtain a paste of homogeneous consistency, which was placed between two sterile gauze pads, to then be applied to the injured skin.

Phytochemical screening

Preliminary phytochemical screening was carried out following Lock's methodology (15). The leaves of *Annona muricata* L. were dried at room temperature, cut into small pieces and soaked in 300 mL of 70% ethanol for 48 h, shaking the container several times a day. The resulting extract was filtered and evaporated on a rotary evaporator at 40 °C. One gram of dry residue was weighed, 30 mL of solvents of different polarities were added to obtain the dichloromethane, ethanolic, aqueous and aqueous acid extracts. Chemical identification, coloration and/or precipitation analyzes were carried out to determine the presence of active principles: steroids and triterpenes (Liebermann-Burchard test), flavonoids (Shinoda test), phenolic compounds (Ferric chloride test), saponins (Foam test), tannins (Gelatin test), alkaloids (Dragendorff, Mayer and Wagner tests), anthocyanidins (Rosenheim test), anthraquinones and naphthoquinones (Borntrager test).

Experimental design

Evaluation of healing activity

The specimens were randomly distributed in four groups of 8 rats each, including the Healthy group, without burns, which received food and water *ad libitum*; the Control group with induced second degree burns and without treatment only received their usual diet for 15 days; the Experimental group with induced burns and treatment with *Annona muricata* L. leaves in the form of a cataplasm (3 g/day) for 15 days and the Standard group with induced burns and treatment with 1% silver Sulfadiazine (3 g/day) for 15 days.

Burn induction

To induce second-degree burn in the control, experimental and standard groups, the specimens were anesthetized with ketamine 87 mg/kg and xylazine 13 mg/kg intraperitoneally (16).

The hair of the dorsal paravertebral region of the experimental specimens was cut and depilated using depilatory cream, after which the depilated area was disinfected and the burn was carried out. For the induction of the second-degree burn, a disinfected 60-watt light bulb was used, which was then kept on for 1 hour. After that, three applications of the hot bulb on the skin of the rats were made, each of the applications lasted 20 seconds with rest intervals of 10 seconds, with which it was possible to affect a good part of the dermis of the burned area, without reaching the subcutaneous area. The macroscopic characteristics of the lesions corresponded to second-degree burns.

Measurement of wounds

Wounds were measured to evaluate the progress of the healing process through the reduction of the burned body area (mm^2) at the lateral and caudal skull level, on days 1, 3, 5, 7, 9, 11, 13, 15 post burn. Each rat was placed in a stable position and the wound margin was traced on a transparent plastic sheet, with a fine-tipped pen. A millimeter plastic ruler was used to make the measurement. Data were recorded on each scheduled day (17).

Histopathological study

The specimens were sacrificed with excess surgical anesthesia the day after the end of the study. Skin tissue samples were taken, making cuts 2 cm long and 2 cm wide around the scar. The extracted tissues were placed in 10% formalin to be taken later for histopathological analysis. Hematoxylin and eosin (H&E) were used to stain the tissue samples.

Statistical

The statistical package SPSS v.22.0 was used for data processing. The variables were analyzed with the ANOVA test with a significance level of 95% (p

<0.05), and the Tukey HSD test. The graphs were made using the GraphPad Prism 7 Demo program.

Ethical considerations

This study was carried out in accordance with the ethical guidelines and regulations established by the Institutional Ethics Committee for Animal Research of the National University of Trujillo, Peru (Regulation, Code and Manual of Ethics Procedures (RCU N ° 361-2018 / UNT) and the guidelines established in the Guide for the care and use of laboratory animals (18).

Results

Phytochemical screening

Phytochemical screening of *A. muricata* L. leaves revealed the presence of important secondary metabolites such as quinones in the dichloromethane extract; the ethanolic extract revealed the presence of alkaloids, flavonoids, steroids, quinones, phenols and tannins; the acidic aqueous extract revealed the presence of alkaloids and flavonoids and the aqueous extract revealed the presence of flavonoids, tannins, alkaloids, steroids and leukoanthocyanidins (Table 1).

Histopathological study

The parameters evaluated in the histopathological study included the presence of collagen, fibroblasts, angiogenesis, macrophage infiltration, and important structural changes. The difference between the damage produced in the control group with respect to healthy skin was evident and also with the experimental groups and the standard group where the reparative processes were influenced by the administered treatments (Figure 1).

Evolution of healing activity

Figure 2 shows the evolution in the reduction of the body area injured by burns in albino rats, where it is evidenced that this reduction in wound size and ongoing healing was very significant ($p < 0.05$) in the experimental group (*A. muricata* L.), which is observed from day 11 of the beginning of the study, becoming more evident on day 13 and 15 compared to the control group. A greater decrease in the size of the burn wounds was found in the experimental group compared to the control group and, in a similar way, it happened with the standard group compared to the control group.

Discussion

As initially defined, burns are the result of damage to the skin caused by excessive heat or chemicals. However, the most common cause is due to exposure to heat. Burns caused by thermal injury are characterized by the initial distribution of heat, thus turning these injuries into dynamic ones, they can advance during the first 2 to 3 days, therefore, a frequent evaluation of the wound is necessary to generate an optimal management of recovery (19).

The healing process of this type of burns is a sequence of processes or phases of the inflammatory response, the immediate or nervous, intermediate or immune and late or endocrine phases can be evidenced, therefore, the intensity, extension and location of thermal insult, intensity and duration of ischemia-revascularization, immune response, coagulation-fibrinolysis, and infection. These processes affect the different components that participate in the healing of the lesion, such as angiogenesis, fibroplasia and epithelialization (20).

In the present study, a method of induction of burns caused by thermal injury was used, with the help of a 60-watt light bulb, a methodology similar to that reported in other studies of induction of second-degree burns in animal models, in which were used different instruments such as metal rods, steel tubes and metal plate, all of them previously heated to a temperature ranging between 80 ° C and 150 ° C, then contact was made with the skin of the previously anesthetized specimens by periods of 1, 15 or 20 seconds, depending on the instrument used (21–24).

Process that can be observed in Figure 1, where the results with respect to the histopathological study are shown. In Fig. 1.A, all the characteristics corresponding to normal skin, such as the superficial layer of keratin, stratum corneum and sebaceous gland; on the other hand, in Fig1.B, which corresponds to the control group, the presence of macrophages is possibly due to the fact that during the inflammatory response of the burn there is a cell death due to apoptosis, therefore, the macrophages eliminate the apoptotic cells by phagocytosis. However, during the healing of burns, the synthesis

and release of PDGF, TGF-β, TGF-α and TNF, produced by macrophages and platelets, among other cells that are responsible for the formation of granulation tissue, increases. The production of these growth factors and cytokines is believed to decrease when epithelialization is complete, which has an inhibitory function of fibrogenesis and angiogenesis (25).

In Fig1.C, in the standard group, a decrease in angiogenesis and fibroblasts can be observed, as well as the presence of macrophages in the dermis; which is indicative of an evolution of the reparative process that is possibly due to the mechanism of action promoted by sulfadiazine or by the silver ion contained in its structure by individual action or in a synergistic way. Likewise, when there is a burn injury, there is a lack of control of cellular and humoral immunity, with alterations in the activation and function of neutrophils, macrophages, T lymphocytes and B lymphocytes. That is why the presence of macrophages is also observed in the figure; in addition to being these, the main producers of pro-inflammatory mediators (26).

Studies realized by Oaks and Cindass; and Nesbitt and Sandman, report the mechanism of the silver ion as an antibacterial agent, since it is capable of binding with bacterial DNA, thus replacing hydrogen bonds of the adjacent nitrogen atoms of purines and pyrimidines. The N-H bond is weaker than the N-Ag bond, causing a failure in bacterial DNA replication. Likewise, the associated sulfonamide intervenes in the synthesis of folic acid as a competitive inhibitor with p-aminobenzoic acid as it has a similar structure. It also inhibits dihydroteroate synthetase, responsible for the precursor of folic acid. Therefore, the sulfonamide has a more selective action than the silver ion, since eukaryotic cells do not synthesize folic acid, but take it from food. Bacteria, on the other hand, need to synthesize folic acid, which is why they are very sensitive to the attack of sulfonamide (27,28).

In Figure1.D, the effects of the *Annona muricata* L. cataplasm is evidenced, finding a reticular-like chorion in fibrous junction, greater presence of collagen, scarce fibroblasts and blood vessels; all of this together are indicative of an intense repair process, an effect probably attributed to the cataplasm. The explanation for this is based on the fact that simultaneously with the granulation tissue, fibroblasts accumulate in the wound when the inflammatory phase is ending; reaching its highest population one to two weeks after injury. By the end of the first week, fibroblasts are the most common cells in the wound; proliferating and migrating from the normal tissue of the wound margins to later deposit in the collagen matrix in the wound. But one of its important tasks is the synthesis of collagen being noticeable by the second and third days after injury, and it is maximum in one to three weeks. Initially the synthesis exceeds the degradation and when there is a balance between the synthesis and the degradation, the maturation phase begins. Likewise, when the end of the granulation phase begins to mark the fibroblasts diminish and begin to undergo apoptosis, converting the granulation tissue from one rich in cells to one that contains mainly collagen (29).

Similar results to those obtained in our study are found in the research carried out by Bayir et al., where the effect of beeswax on induced burns in a murine model was evaluated. The presence of fibroblasts and the keratinization produced were also reported in the histopathological study, which is an indication that the natural product analyzed would be promoting the skin regeneration process and optimizing the healing process, these indications are also evidenced in the Figure 1.D (30).

In Figure 2, a significant reduction of the burned body area is distinguished in the experimental and standard groups, these observations can be based on the presence of antioxidant bioactive agents in the cataplasm of *A. muricata* L. leaves, such as flavonoids, tannins, alkaloids, among other secondary metabolites found in the phytochemical study of *A. muricata* leaves (Table 1). One of the first stages of healing in thermal injuries occurs with inflammatory processes, in which the area of direct contact with the heat source, the coagulation area, provides a favorable extracellular matrix for cell migration mediated in part by the presence of Platelet-derived growth factor, vasoactive mediators, and chemotactic factors generated by cells of the injured parenchyma. Thus; the recruitment of inflammatory cells to the injury site is a fundamental factor in the transition between inflammation and tissue repair (31).

Flavonoids exhibit antioxidant action mechanisms that include the suppression of ROS formation

through the inhibition of enzymes or the chelation of trace elements involved in the generation of free radicals; also, ROS uptake and positive regulation or protection of antioxidant defenses. Flavonoids also inhibit the enzymes involved in ROS generation, like as, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, among the most important actions (32).

Therefore, the mechanism and sequence of the fact by which free radicals interfere with cellular functions in the skin layers appears to be lipid peroxidation, resulting in cell membrane damage. This cell damage causes a change in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death. Free radicals can attract various inflammatory mediators, contributing to overall inflammatory response and tissue damage. Increased production of reactive oxygen species during injury resulting in the consumption and depletion of endogenous scavenging compounds (superoxide dismutase, catalase, peroxidase, and glutathione, but also non-enzymatic homologues such as glutathione, ascorbic acid, and α-tocopherol). Flavonoids may have an additive effect on endogenous scavengers and antioxidants (33).

In some studies, they have identified active secondary metabolites such as arabionolactan, naphthoquinones, linoleic acid methyl-ester, β-sitosterol, and the flavonoids; that directly and positively influence the stabilization and acceleration of the healing process. Flavonoids are claimed to support tissue regeneration after damage, requiring the coordinated action of a large number of biochemical systems, the nature of which depends on the presence or absence of toxin contamination in the wound (34).

Annona muricata L leaves, administered topically, in the form of cataplasm, revealed a healing effect on second-degree burns. Therefore, this plant could be a very important source to obtain products for burns treatment. Additional studies are required to elucidate its mechanism of action and determine the exact active principles responsible for the healing effect.

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Table 1. Phytochemical screening of the leaves of *Annona muricata* L.

		Extracts of *Annona muricata* L.			
Phytoconstituents	**Test**	**DM**	**E**	**AA**	**A**
	Mayer	-	+	++	++
	Wagner	-	+	++	++
Alkaloids	Dragendorff	-	++	++	++
Flavonoids	Shinoda		+++	-	+++
Steroids	Liebermann-Burchard	-	++	-	+
Quinones	Borntrager	+++	++	-	-
Phenols	Ferric chloride	-	++	-	++
Tannins	Gelatin	-	+++	-	++
Leukoanthocyanidins	Rosenheim	-	-	-	+++
Saponins	Foam	-	-	-	-

DM: Dichloromethane, E: Ethanolic; AA: Aqueous acid; A: Aqueous
Presence and intensity: (-): absence; (+): few; (++): moderate; (+++): abundant

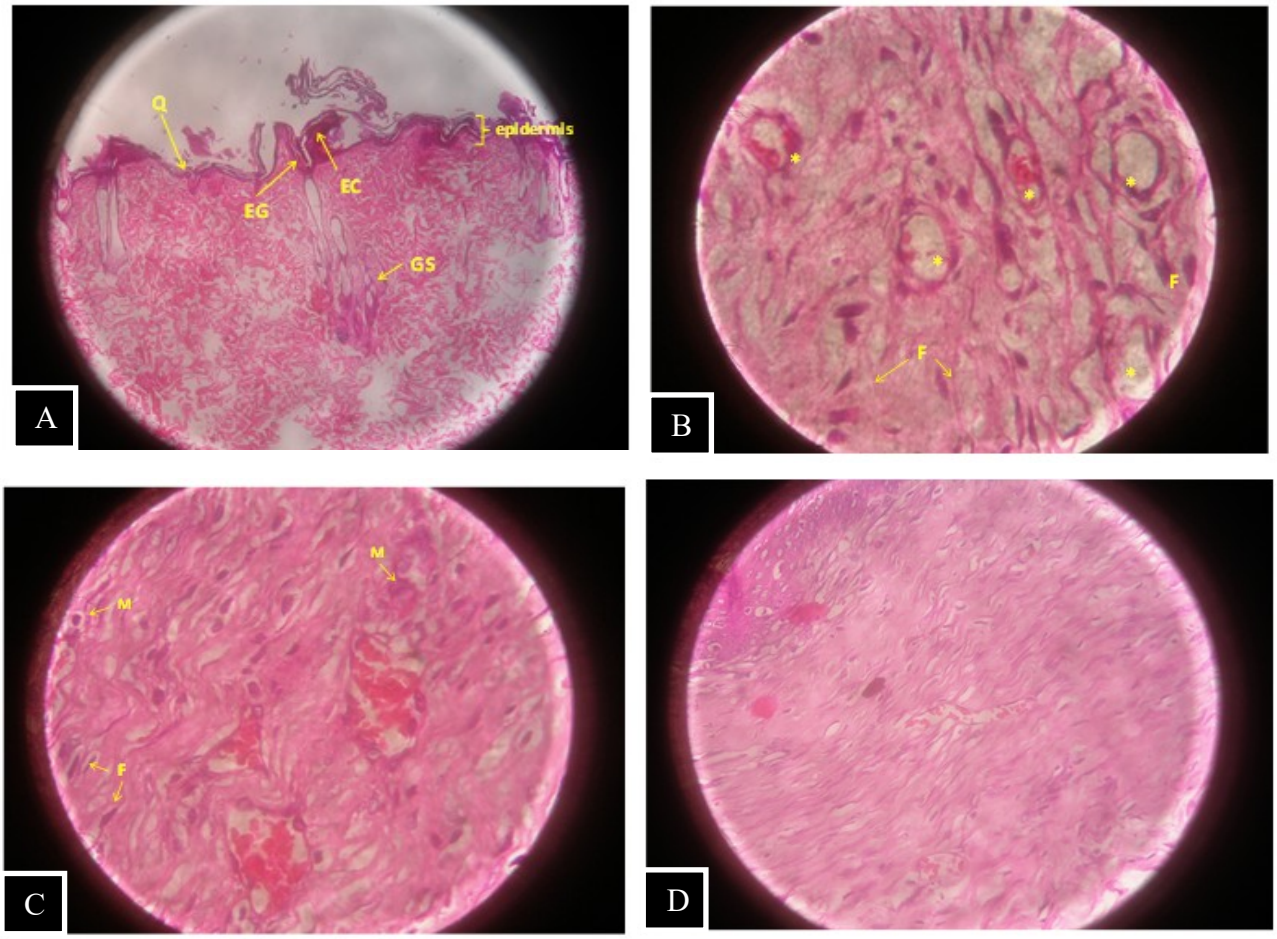

Figure 1. Microphotographs of rat skin. **A. Healthy Group**. normal skin. Perpendicular cut. The superficial layer of keratin (Q), stratum corneum (EC), the basal layer of the germinal stratum (EG) forms papillae meshed with the chorion. Sebaceous gland (GS). All characteristics of normal skin. H&E 100x. **B. Control Group**. Chorion detail. Abundant stratum in thick and fine fibers that correspond to collagen and fibroblasts (F), there is an increase in blood vessels (*) that corresponds to angiogenesis. The vertical arrangement of the fiber bands is observed. Macrophages are present. These findings correspond to granulation tissue in full reparative healing. H&E 100x. **C. Standard Group** (Silver sulfadiazine). Dermis: Decreased blood vessels and fibroblasts (F), presence of macrophages (M) which indicates an evolution of the reparative process. H&E 400x. **D. Experimental group** (*Annona muricata* L.). Chorion with a reticular appearance in fibrous junction, greater presence of collagen and few fibroblasts, few blood vessels, indicative of intense repair. H&E 400x.

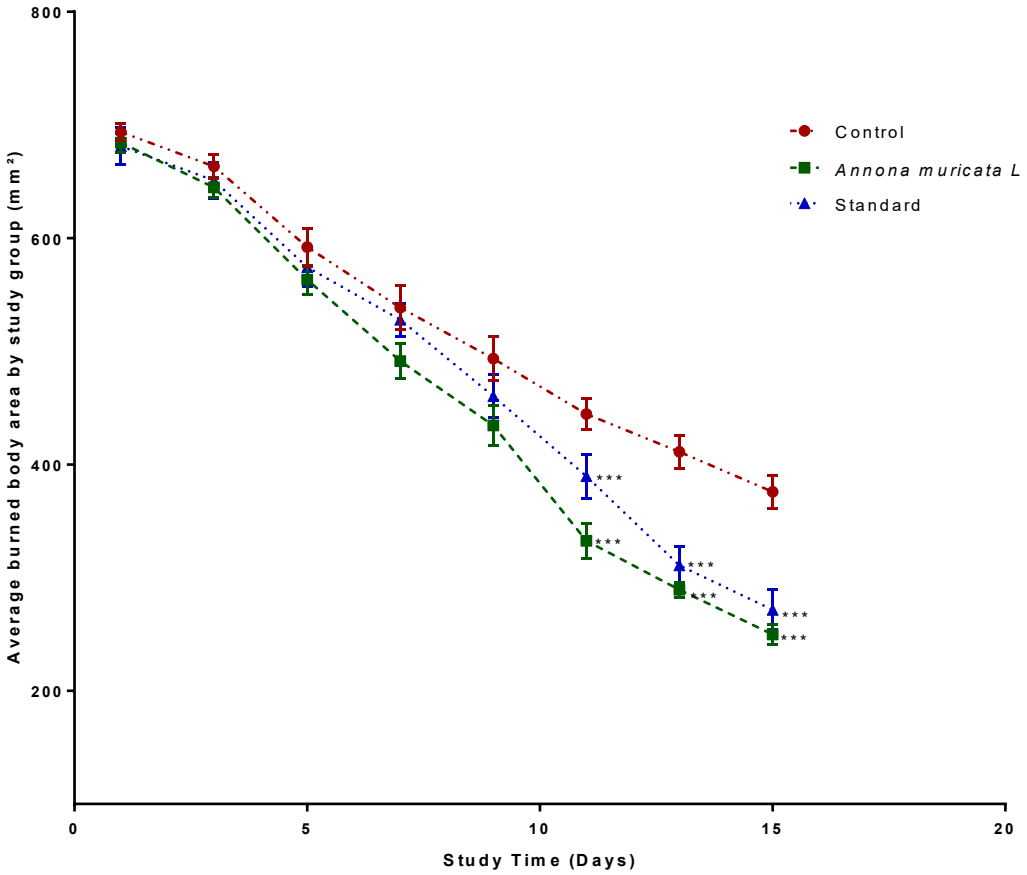

Figure 2. Variation of the average burned body area (mm^2) during experimentation time in rat from the control, experimental (*Annona muricata* L.) and standard (1% silver sulfadiazine) groups. The values correspond to the mean ± standard error of the mean, n = 8, (*** p <0.05)