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Trace of chestnut on platelets proliferation in male rats: In vivo study

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Abstract

The study seeks the efficacy of the chestnut extract on platelet proliferation and its function in guaranteeing more tangible physiological information about unexpected mortality caused by thrombus formation. Twenty-eight male adult rats were organized into two sets, each with 14 rats, at two intervals (14 and 35 days): the untreated set and the set that received chestnuts (600 mg/kg body weight) orally. Fourteen days after treatment, fourteen rats perished. The remaining half of the animals were left and then allocated into two sets in the untreated and treated groups with chestnuts at the same dose for 35 days. Findings demonstrate a significant reduction in thrombopoietin following 14 days of chestnut administration, as well as platelet counts at 14 and 35 days comparable to untreated rats, while hikes up hormone level and P-LCR at the 35th day of treatment; however, clotting time at both intervals prolonged about untreated rats. Between two intervals, chestnuts increased thrombopoietin levels, clotting time, and platelet count at 35 days, compared to the same group at 14 days of treatment. Immunohistochemistry at 14 and 35 days revealed that IL-3 was expressed strongly in the untreated rats' bones but only moderately in the chestnut group. Furthermore, at two intervals, rat's sans treatment exhibits robust VEGF expression in their aorta, whereas rats with chestnut extract dosages showcase weak expression. As a recap, chestnut hinders platelet proliferation by interfering with thrombopoietin, thrombocyte numbers, and IL-3 and acting as an anti-angiogenetic and anti-thrombogenic substance.

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Introduction

A thrombus is an accumulation of platelets and tissue fibrin that may accumulate in a body vessel, where it can either entirely or partially obstruct blood flow under certain circumstances. It is potentially fatal if neglected or untamed, just like the illnesses connected to canine and feline respiratory thromboembolism and bilaterally hindlimb numbness in the horse-caused recumbency may result from the distal aorta being completely blocked by thrombotic clots (1). Chestnut is a species of *Aesculus hippocastanum L.*, frequently called horse chestnut, and an ancestor of the family known as Fagaceae. Castanea is a genus of ten species that reside in southern Europe, northern Africa, Asia, and the eastern United States. The most prominent chestnut-producing continent in Europe exists in Italy (2). Chestnut agriculture has furnished them with palatable fruits and robust wood, whereas in previous years, the acronym "chestnut" was utilized as a synonym for the tree itself and the fruits (3). Total sugar, saturated and unsaturated fatty acids, crude fat, fiber, protein, ascorbic acid, and polyphenols are nutritional and chemical constituents of chestnuts (4). The phytochemical descriptions, flavan-3-ol compounds that gave antioxidant efficacy inside the cells versus hydroxyl and superoxide radicals, as well as peroxynitrite, and the nutraceutical nature of chestnut cultivars, were researched in up-to-date research by Chang and colleagues in 2020, they concluded that the total amount of phenolic compounds varied between 42.8 and 58.6 mg/100 g weight (5). Horse chestnut herb extract, which has anti-inflammation, antiedematous, and coagulability properties, contains the active phytoconstituents aescin and is used as a remedy for persistent venous inadequacy (6). Based on certain reports, horse chestnut has long been utilized as feed for livestock and as an essential provider of forage for animals; in addition, it has the potential to improve feeding effectiveness and as an element of the eating habits of several Americans (7). On the flip side, coumarin, a component of chestnut, often interferes with blood-thinning results (8), whereas procyanidins have been shown to prevent aggregation of the platelets by adenosine diphosphate (ADP) (9). Also noteworthy is the most recent finding, which demonstrates that escin (the major active constituent of *Aesculus hippocastanum*) inhibits angiogenesis by suppressing the production of vascular endothelial growth factor (10). It is important to note that IL-3 acts as a growth inducer and regenerates platelets by developing megakaryocytes from pluripotent hematopoietic stem cells (PHSC) in the bone marrow (11-13).

This study is intended to investigate the effectiveness of chestnut in vivo by measuring thrombopoietin hormone, which affects platelet proliferation, and IL-3, a growth inducer of platelets from bone, and its effect on platelet count and vascular endothelial growth factor, as well as platelet function as measured by clotting time.

Materials and methods

Ethical approval

The institutional committee on ethics at the University of Mosul's, College of Veterinary Medicine embraced all rats' specimens as long as they complied with its code of ethics number. UM.VET.2023.018.

Laboratory animals used in the research endeavor

In the animal breeding facility of Mosul University's College of Veterinary Medicine, Wistar albino male rats were bored, reared to maturity, weighed 150-200 g, and kept in cages at the experimental animal facility of the animal's residence, where they were maintained under a 12-hour light/dark cycle at room temperature and in an air-conditioned space. The animals gained a standard rat diet and unlimited amounts of water during their two-week adjustment period; the trial lasted from November 2022 to April 2023 (i.e., from birth to the end of the experiment).

Protocol for experiment

Twenty-eight male adult rats were seeded into two groups: the untreated group and the treated with chestnut (600 mg/kg body weight) (6) orally (p.o.) group. Fourteen days after treatment, half of the rats (14) perished with ether, and the specimens were collected. However, the remainder of the animals were treated with chestnut and the same dose for 35 days before being sacrificed to obtain the specimens the chestnut powder obtained from (MIAVIT, Germany).

Tests conducted following the drawing of blood

After two intervals (14,35) days of daily oral administration of the chestnut extract, blood specimens (2ml) were taken from the animals' eyes using an ocular poke by a capillary tube. They were put in a gel tube, centrifuged to obtain pure serum, and then frozen at –26°C in the refrigerator for thrombopoietin estimation using the Rat TPO (thrombopoietin) ELISA Kit (ELK Biotechnology, China). A stopwatch is started, and two to three more drops of fresh blood are placed on the glassy slide to calculate the clotting time. At intervals of 15 seconds, a pointed object was pushed across the blood up to strings of fibrin, which was apparent. The time on the watch was promptly stopped, and the clotting time was indicated (12). By heparinized tube and auto hematology analyzer; RT-7600 for veterinary utilized to analyze blood for platelets criteria (13,14).

Immunohistochemistry of specimens

Under stroke anesthesia, rats were killed, their femurs were pulled out, and their hearts were taken to the aorta needed and saline-flushed, then immersed in 10 percent neutral buffered formalin up to the immunohistochemical (IHC) technique for finding out about IL-3 in the bone and VEGF in the aorta.

Bone preparation

The bone sections employed in the immunocytochemistry procedure were dewaxed in xylene, hydrated in ethanol, and then wiped in a saline solution with phosphate buffer saline (PBS). The peroxidase output was then restricted for 30 minutes by putting in a three-percent hydrogen peroxide-methanol mixture. Following that, the sections were preserved at 25°C over 60 minutes. Subsequently, each section underwent incubation for an entire night at four degrees Celsius with elementary antibodies (polyclonal IL-3 Ab 1:200) (E-AB-13273, Elabscience, USA). After three washings, the cut sections were incubated with anti-rats IL-3 for sixty minutes at 37°C. Then, these sections were identified using an avidin-biotin intricate following another three washings. Immunolabeling was done, and then, upon sixty seconds of hematoxylin staining, the sections were rinsed with distal water, dehydrated, and covered to be examined under a microscope (15).

Aorta preparation

Aorta sections made of paraffin had their paraffin removed on charged slides, dried with a grading concentration of alcohol, and washed with PBS. The slides were incubated in 0.3 percent hydrogen peroxide to inhibit peroxidase for thirty minutes. After that, a blocking solution is applied for an hour. The slides were incubated with the initial antibodies for an entire night in the refrigerator. Rat polyclonal VEGF Ab-3 (diluted at a percent of 1:200), which served as the initial antibody, for half an hour, the additional antibodies (E-AB-63481, Elabscience Kit, USA) were diluted within PBS. The sections used as a negative control were incubated in PBS without an initial antibody. The slides then underwent incubation for a half hour with the Kit from the conjugation of streptavidin alongside horseradish peroxidase within a buffer of Tris-HCl containing 0.015% sodium azide. Finally, immunolabeling (3,3′-diaminobenzidine DAB chromogenic substrate) and hematoxylin re-staining were performed on the slides. By applying the Olympus digital camera and a 600x magnifying glass, the expression of VEGF in response to polyclonal antibodies that react strongly was observed (16,17).

Statistical analysis

Sigma Plot (14.0), a static analyzing program, was applied to assess the data, and two-way analysis of variance (ANOVA) was used to determine if there was significant variability at the probability level less than or equal to 0.05 among the two sets and between the two intervals of rats' ages (18).

Results

The data reveals significant (P≤0.05) distinctions among the treated and untreated groups. The oral administration of chestnut lessened thrombopoietin levels within the serum, contrasting with the untreated set at the first 14 days of treatment, while rats given chestnut continuously for 35 days had significantly (P≤0.05) higher levels of the hormone than rats in the untreated set. The analytic comparison of two treatment durations illustrates that the handled set with chestnut the second time raises significantly (P≤0.05) more thrombopoietin compared to the first, whereas the control sets in the two durations show no statistical difference (Table 1). The results in table 2 point to statistically (P≤0.05) prolonged clotting time along with offering chestnut compared with an untreated collection and at two distinct times, whereas chestnut dosage at 35 days significantly (P≤0.05) lengthened blood clotting time about the 14-day treatment duration, with no statistically significant variance in the untreated set. The platelet criteria in table 3 express a significantly (P≤0.05) lower platelet count in the chestnut-treated set over the two intervals of time compared to untreated animals, while there is no variation in the platelet numbers at the two durations. The platelet-large cell ratio (P-LCR) improved significantly (P≤0.05) after 35 days of oral chestnut treatment in contrast to untreated rats, but there was no statistically significant (P≤0.05) distinction between the experimental groups at 14 days. About the two durations, the P-LCR significantly (P≤0.05) increased at 35 days as opposed to 14 days of treatment with chestnut, whereas the rats that did not receive chestnut did not show statistical variability.

Table 1: The repercussions of chestnut on thrombopoietin (pg/ml) serum level in male rats

14 days		35 days	
untreated set-1	Treated set with chestnut-1	untreated set-2	Treated set with chestnut-2
53.93±1.23 aA	45.39±3.48 bB	53.16±2.96 bA	88.55±2.71 aA

*The distinction between both sets is denoted by small letters, whereas the distinction between two-time intervals is denoted by capital letters at P less than 0.05.

Table 2: The repercussions of chestnut on clotting time (minute) in male rats

14 days		35 days	
untreated set-1	Treated set with chestnut-1	untreated set-2	Treated set with chestnut-2
1.70±0.20 bA	5.80±0.20 aB	1.80±0.25 bA	7.60±0.21 aA

*The distinction between both sets is denoted by small letters, whereas the distinction between two-time intervals is denoted by capital letters at P less than 0.05.

Table 3: The repercussions of chestnut on platelets criteria in male rats

Parameters	14 days		35 days	
	Untreated set -1	Treated with chestnut -1	Untreated -2	Treated with chestnut -2
PLT (10^9/L)	654.80±24.83 aA	499.00±24.94 bA	638.80±17.3 aA	482.40±24.35 bA
MPV (fL)	6.78±0.16 aA	7.08±0.05 aA	6.80±0.11 aA	6.76±0.13 aA
PDW (fL)	11.64±0.25 aA	11.76±0.34 aA	11.56±0.30 aA	11.12±0.35 aA
PCT (%)	0.39±0.04 aA	0.40±0.06 aA	0.38±0.03 aA	0.31±0.06 aA
P-LCR (%)	4.50±0.34 aA	4.46±0.25 aB	4.67±0.22 bA	5.46±0.19 aA

*At P less than 0.05, the distinction between both sets is denoted by small letters, whereas the distinction between two-time intervals is denoted by capital letters.

Figures 1 demonstrate the immunohistochemistry staining of the rats' bones, revealing interleukin-3's strongly positive expression in untreated rats, respectively. However, interleukin-3 showed moderate expression after 14 days of chestnut treatment at the same magnification power as the untreated rats. Moreover, after 35 days of chestnut administration, the level of IL-3 expression endures moderate, whereas in untreated rats, the expression remains strong (Figure 2). After handling the specimens with specific Ab for VEGF expression in the endothelial cells of the aortic artery, findings show that in untreated animals and following 14 and 35 days of starting experiments, there is strong expression. In contrast, when chestnut is orally given, there is a weak expression of VEGF (Figures 3 and 4).

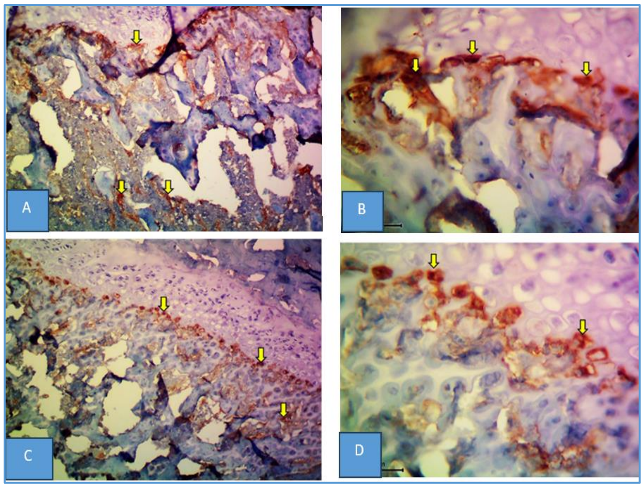

Figure 1: Microscopy photo of immunohistochemistry in the untreated set (A, B) at 14 days; arrows show strong positive expression (+++) of IL-3 in the bone. In the treated set (C, D), arrows show moderately positive expression (++) of IL-3 in bone (A, C: 100X), (B, D: 400X), (scale-bare = 50µm).

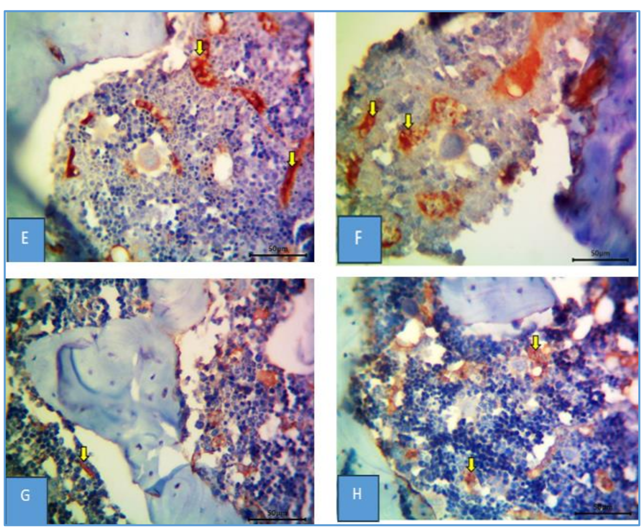

Figure 2: Microscopy photo of immunohistochemistry in the untreated set (E, F) at 35 days; arrows show strong positive expression (+++) of IL-3 in the bone. In the treated set (G, H), arrows show moderately positive expression (++) of IL-3 in bone (E, G: 100X), (F, H: 400X), (scale-bare = 50µm).

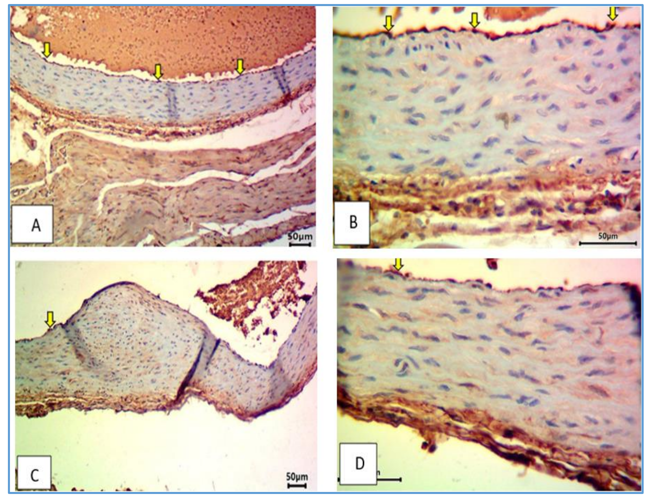

Figure 3: Microscopy photo of immunohistochemistry in the untreated set (A, B) at 14 days; arrows show strong positive expression (+++) of VEGF in endothelial cells of the aorta. In the treated set (C, D), arrows show weak positive expression (+) of VEGF in endothelial cells of the aorta (A, C: 100X), (B, D: 400X), (scale-bare = 50µm).

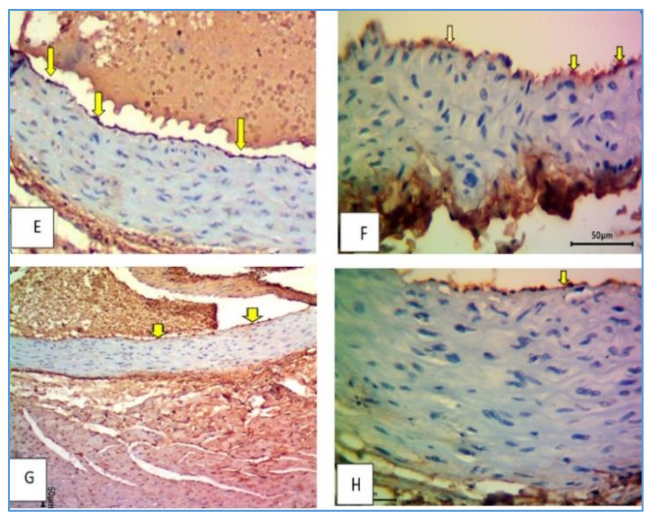

Figure 4: Microscopy photo of immunohistochemistry in the untreated set (E, F) at 35 days; arrows show strong positive expression (+++) of VEGF in endothelial cells of the aorta. In the treated set (G, H), arrows show weak positive expression (+) of VEGF in endothelial cells of the aorta (E, G: 100X), (F, H: 400X), (scale-bare = 50µm).

Discussion

A private data inspection revealed from recent research that 14 days of chestnut use resulted in a decrease in thrombopoietin, along with platelet counts (PLT) at 14 and 35 days while raising hormone levels and platelet-large cell ratio (P-LCR) on the 35th day of treatment beside prolong the time required for clotting. As an outcome, my findings are consistent with those of specialists who have discovered that natural supplements, for example, feverfew, ginger root, garlic, ginseng plant, ginger, and horse chestnut, lessen platelet formation and assemblage, two processes that are crucial to avoiding thrombosis and keeping hemostasis, and reflect on the count of platelets (19). The results line up with the researchers finding that the bark of chestnut has been shown to alleviate reactive platelets and the sequence of coagulation events in blood plasma (20). At the same time, other scholars team figured out that the extract of chestnut and its fundamental ingredients (Coumarins) can partially prevent undesirable clotting sequences by shielding fibrinogen from the nitrative wreckage triggered by peroxynitrite; further investigation is needed to determine the precise mechanism of action (21). Because the extract inhibits platelet proliferation, it also actually inhibits thrombopoietin, which impacts all facets of platelet generation, from self-renewal via promotion of the development of megakaryocyte precursor cells in aid of the transformation of these cells into platelet-producing cells (22). This conclusion is compatible with current results after 14 days of treatment. However, the cause led to elevated hormone levels after 35 days of dosage extract harmonious with Kuter and Gernsheimer demonstrated that the rate of platelet production is inversely related to the circulating TPO level: when platelet production is low, less TPO is cleared, and levels rise; when platelet production is high, more TPO is cleared, and levels fall (23).

To ensure that the results are properly interpreted and discussed, the following platelet indices were used: platelet crit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), platelet-large cell ratio (P-LCR), and platelet count, which are considered biomarkers and indicators of the activity of thrombocytes and also belong to the morphological concepts and the proliferation kinetics of platelets (24,25). Some platelet criteria, such as MPV, PDW, and PCT, showed no discernible differences between the treated and untreated groups even after two interval periods except P-LCR and platelet count. Even though these parameters indicate platelet reactivity and are biomarkers for several inflammatory conditions (26), there were no noticeable differences between groups because the animals were in good health. The previous study outlined a direct correlation between P-LCR MPV and PDW and a reverse correlation with the number of platelets in thrombocytopenia, which seems more vulnerable to fluctuations in the size of thrombocytes (27). Moreover, it has been concluded that a large platelet percentage reflects a population of immature platelet cells; therefore, the P-LCR has a high predictive assessment efficacy and may detect variations in thrombocyte size (28). I suspect the adverse feedback process by chestnut on thrombopoietin at 35 days triggered a hike in P-LCR, leading to thrombocytopenia and the emergence of large platelets that indicated immature cells. Likewise, platelets being activated affects several variables, one of which is the Vascular Endothelial Growth Factor (VEGF), and consequently affects angiogenesis (28). Some specialists have declared that platelets and white blood cells work together to transfer angiogenic factors, such as VEGF, and induce vascular tubes to develop in vitro (29). Thus, platelets signify proangiogenic function in vivo in the physiological concept (30). After all of this evidence, I was convinced because chestnuts, depending on the study's information gathered, caused a drop in the number of platelets along with a weak expression of VEGF in the aorta, which will indirectly dampen platelet activity and function. Interleukin -3 governs numerous facets of the colony-stimulating hematopoietic stem cell as a growth inducer and the development of megakaryocytes (31-34). A particularly powerful cytokine, together with interleukin-3, that physiologically monitors the creation of platelets precisely and possesses pleiotropic impacts on hematopoiesis generally is thrombopoietin, also known as megapoietin and a factor for growing megakaryocytes. Therefore, there is a strong correlation between interleukin three and thrombopoietin's platelet regulation (35). Additionally, a recent study demonstrated that when a platelet-rich plasma mixture was infused into the joint's capsule, there was an increase in the amount of newly created chondrocytes as a result of the platelets' release platelet-derived growth factors, which affect the growth and maturing of the cells alongside matrix metabolizing stimulators (36). Likewise, these growth factors operate as chemotactic substances that platelets release to contribute to angiogenesis (37). Observations of the current investigation show that IL-3 expression diminishes, which in turn lessens platelet production, thereby confirming all of the implications triggered by chestnut. Likewise, the plasma concentrations of angiogenic substances reveal a synergistic interaction between VEGF-A and platelets (38). It also links VEGF to clotting and anticlotting proteins (39). Finally, thrombin being subjected causes signals that regulate VEGF transcription of genes and then trigger angiogenesis in the microvascular; therefore, the results above provide improved knowledge of vascular responses when faced with thrombi, which may aid in interpreting the findings of experiments addressing issues linked to damage to endothelial cells and coagulation disorders (40), This outcome is in agreement with own investigation, which pointed out that chestnut treatment lengthened the time for clotting and was responsible for a reduction in vascular endothelial growth factor expression in the aorta.

Conclusions

It can be inferred that chestnut extract has a slump impact on thrombopoietin levels in the circulation and IL-3 inadequate expression in the marrow of the bones, with an abundance of underdeveloped platelets symbolized by P-LCR. All of the above outcomes display a drop in the count of platelets, pointing out that the chestnut participates in an antiplatelet impact. Furthermore, the chestnut lengthens the time needed for clotting, indicating that it is an anticoagulant agent. Lastly, chestnuts hamper VEGF expression, suggesting that they have an anti-angiogenic effect.

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Conflicting interest

The researcher claims there are no conflicts of interest about the publication of this work.

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أثر الكستناء على تكاثر الصفيحات الدموية في ذكور الجرذان: دراسة في الجسم الحي

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الخلاصة

لضمان المزيد من المعلومات الفسيولوجية الملموسة حول الوفيات المفاجئة الناجمة عن الخثرة، سعت الدراسة إلى معرفة فعالية مستخلص الكستناء على تكاثر الصفائح الدموية ووظيفتها. ٢٨ من ذكور الجرذان البالغة نظمت في مجموعتين منفصلتين، تحتوي كل منهما على ١٤ جرذ، لفترتين زمنيتين (١٤ و٣٥ يوما): المجموعة غير المعاملة والمجموعة التي أعطيت لها الكستناء (٦٠٠ ملجم/كجم من وزن الجسم) عن طريق الفم. وبعد ١٤ يوما من المعاملة، قتل أربعة عشر جرذا. تم ترك النصف المتبقي من الحيوانات ثم تقسيمها لمجموعتين في المجموعة غير المعالجة والمعاملة بالكستناء بنفس الجرعة لمدة ٣٥ يوما. أظهرت النتائج انخفاضا معنويا في الثرومبوبويتين بعد ١٤ يوما من المعاملة بالكستناء، بالإضافة إلى إعداد الصفائح الدموية عند اليوم ١٤ و٣٥ مقارنة بالجرذان غير معاملة، مع الزيادة في مستوى الهرمون ونسبة الصفائح الدموية الكبيرة في اليوم ٣٥ من المعاملة؛ بينما انخفض الوقت اللازم للتخثر بالمقارنة في كلا الفترتين بالمقارنة مع الجرذان غير المعاملة. بين الفترتين الزمنيتين، رفع الكستناء مستوى الثرومبوبويتين والوقت اللازم للتخثر ونسبة الصفائح الدموية الكبيرة خلال ٣٥ يوما، مقارنة بنفس المجموعة عند ١٤ يوما من المعاملة. كشفت الكيمياء المناعية في ١٤ و٣٥ يوما أن الانترلوكين الثالث تم التعبير عنه بقوة في عظام الجرذان غير المعاملة ولكن بشكل معتدل فقط في المجموعة المعاملة بالكستناء. علاوة على ذلك، في الفترتين الزمنيتين، أظهرت الجرذان غير المعاملة تعبيرا قويا عن عامل نمو بطانة الأوعية الدموية في الأبهر، في حين أظهرت الجرذان التي جرعت بمستخلص الكستناء تعبيرا ضعيفا. كخلاصة، يعمل الكستناء على كبح تكاثر الصفائح الدموية عن طريق تأثيره في الثرومبوبويتين، وأعداد الصفائح الدموية، والانترلوكين الثالث، بالإضافة إلى كونه مادة مضادة لتولد الأوعية الدموية ومضادة للتخثر.