

A topical formulation of *Anacardium occidentale* L. leaves extract enhances wound healing via mediating TNF- α and TGF- β

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Repeated trauma, inadequate perfusion or oxygenation, excessive inflammation, infection, oxidative stress, and diabetes all contribute to impaired wound healing. In a clinical setting, delay in healing may result in severe complications such as gangrene, leading to extended hospitalization, amputation and even death of the patient. Slow wound healing and limitations of currently available synthetic medication, urges the need for a safe, affordable, patient-friendly, and multi-modal herbal wound care agent. *Anacardium occidentale* L., commonly known as cashew and locally, Kaju, is reported to possess antidiabetic, antibacterial, antioxidant, anti-inflammatory and antiulcerogenic activities. In this study, we evaluated the wound healing activity of gel containing the extract of *A. occidentale* leaves in rats using incision, excision and dead space wound models. Increased wound contraction, breaking strength, hydroxyproline and hexosamine content, TGF- β level and decreased level of TNF- α indicated that the developed gel accelerated the wound healing process. Moreover, the docking studies indicated that the aqueous alcoholic extract exerted healing activity by inhibiting GSK3- β through β -catenin dependent Wnt signaling pathway. Thus, it can be concluded that the *Anacardium occidentale* leaves extract can be a potential wound healing agent by acting on various phases of healing process.

Keywords: Cashew tree, Glycogen synthase kinase-3 β protein (GSK3- β), Growth factor, *In silico* molecular docking, Kaju, Pro-inflammatory cytokine, Wnt signaling pathway

Wound healing is a complex process composed of various interconnected and overlapping phases such as haemostasis and inflammation, proliferation, migration of different cell types, neovascularization, granulation, re-epithelialization and remodeling¹. It is coordinated by a complicated signaling system involving various growth factors, cytokines and chemokines². Growth factor such as TGF- β stimulates angiogenesis, proliferation of fibroblasts, differentiation of myofibroblasts, and formation of extracellular matrix³. The acute inflammatory phase involves the secretion of various inflammatory mediators, such as cytokines and growth factors that regulate the inflammatory phase of wound healing via cell proliferation, chemotaxis and connective tissue formation. The persistent high levels of these mediators account for the delay in the granulation tissue formation and failure of wound closure⁴. Hence, estimation of the level of growth factor

(TGF- β) and inflammatory cytokine (TNF- α) in wound tissue is essential to monitor the wound healing process. It has been proven that the Wnt/ β -catenin pathway can enhance wound healing through the inhibition of glycogen synthase kinase-3 β protein (GSK3- β), an important regulatory protein⁵. Previous studies demonstrated the role of *in silico* docking studies in ensuring the GSK3- β inhibitory activities in correlation to *in vivo* wound healing activity⁶.

Plants have immense potential in wound management and treatment. Phytoconstituents of the plants such as tannins⁷, flavonoids⁸, triterpenoids⁹ and alkaloids¹⁰ have been found to affect one or more phases of the wound healing process. Hence, herbal therapy emerges as an alternative strategy for the treatment of wounds¹¹.

Anacardium occidentale L. (*Anacardiaceae*), commonly known as Cashew and locally Kaju, occurs in South America, Africa, India, Sri Lanka and Philippines. *Anacardium occidentale* leaves have been reported to possess various pharmacological activities such as antimicrobial¹², antioxidant¹³,

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hypoglycemic¹⁴, anti-inflammatory¹⁵ and antidiabetic effect on neonatal streptozotocin diabetic rats¹⁶. The results of the preliminary study carried out in our laboratory showed promising antioxidant activity, antimicrobial activity against reported wound pathogens and the presence of phytoconstituents such as gallic acid, methyl gallate, ethyl gallate and quercetin were identified in the aqueous alcoholic extract of *Anacardium occidentale* leaves by HPLC methods¹⁷.

Although several pharmacological studies of *Anacardium occidentale* L. have been reported, no systematic study has been carried out on the wound healing activity of this plant yet. Here, we investigated the wound healing activity of the aqueous alcoholic extract of *A. occidentale* leaves on fresh wounds in albino rats employing excision, incision and dead space models. The mechanism of action was also investigated in terms of effective docking of the phytoconstituents present in bioactive extract against selected target GSK- β and estimating the level of inflammatory cytokine (TNF- α) and growth factor (TGF- β).

Materials and Methods

Materials

Carbopol 971 P NF was purchased from Lubrizol Life Sciences (Mumbai, India). D(+) glucosamine HCl was purchased from E Merck Pvt. Ltd. (Mumbai, India). L-hydroxyproline was purchased from Sigma-Aldrich Chemicals Co., (St. Louis Mi, USA). Betadine (Win Medicare Pvt. Ltd.) ointment was purchased from the local medical store. Rat TGF β ELISA kit and rat TNF- α ELISA kit were purchased from Wuhan Fine Biological Technology Co., Ltd. (China). All other chemicals and solvents used were of analytical grade.

Plant material and preparation of the extract

Anacardium occidentale leaves were collected from Yeoor forest, Thane, India. The plant material was authenticated at Agharkar Research Institute, Pune, Maharashtra with a voucher specimen no. 3/187/2015/Adm-2799/Auth 15-170.50 g of the powder was extracted with solvents of different polarities such as petroleum ether (60-80°C), chloroform, methanol, aqueous alcohol (50:50) and water using Soxhlet extraction apparatus for 30-32 h. The percentage yield of extract was determined by extracting 10g of powdered material with 100 mL

solvent. The obtained extracts were concentrated in a water bath and the yield of the extracts obtained was calculated.

Preliminary phytochemical screening of extracts

One gram of each extract of plant material was dissolved in 100 mL of water or methanol and filtered to obtain a stock of concentration of 1% (v/v). The filtrate thus obtained was used to identify the presence of various phytochemical constituents¹⁸.

Determination of total phenolic content (TPC)

The total phenolic content was measured using the Folin-Ciocalteu reagent based on procedures described by Al-Dalahmeh *et. al.* with some modifications¹⁹. Briefly, 1 mL of extract solution (1 mg/mL) or (0.1 mg/mL) was added to 0.5 mL of Folin Ciocalteu reagent and 5 mL of distilled water. The mixture was incubated at room temperature for 10 min. Then 1.5 mL of anhydrous sodium carbonate solution (10% w/v) was added and the final volume was made up to 10 mL. The final mixture was allowed to stand at room temperature (30°C) for 2 h with intermittent shaking. Then the absorbance of the dark blue colour that developed was measured at 725 nm using a UV-Vis spectrophotometer. The experiment was carried out in triplicates. Gallic acid was used for preparing the standard curve (10 μ g/mL to 100 μ g/mL). The total phenolic content in the plant extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract.

Determination of total flavonoid content of extracts

The total flavonoid content of all prepared extracts was estimated by the aluminium chloride colorimetric method²⁰. An aliquot (1 mL) of each extract or standard solution of catechin (50, 100, 150, 200, 250 and 300 mg/L) was mixed with 4 mL of distilled water and subsequently with 0.3 mL of 5% NaNO₂ solution. Post incubation of 6 min, 0.3 mL of 10% AlCl₃ solution was added and then allowed to stand for 6 min, followed by adding 2 mL of 1M NaOH solution to the mixture. Immediately water was added to the sample to bring the final volume to 10 mL, the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the pink colour was determined at wavelength 510 nm. Total flavonoid content was expressed as mg rutin equivalents per one gram of dry extract (mg RE/g of extract). All measurements were carried out in triplicates.

Table 1 — Formulation of batches of gel formulations

Ingredients	F1	F2	F3
Carbopol 971 P NF	1%	1.5%	2%
AAE-AOL	1%	1%	1%
Ethanol	4 mL	4 mL	4 mL
Propylene Glycol	2.5 g	2.5 g	2.5 g
Triethanolamine	q.s to neutralise gel base		
Distilled water	q.s upto 50 g		

[AAE-AOL, Aqueous alcohol extract of *Anacardium occidentale* leaves]

Drug formulation

About 1% of aqueous alcoholic extract of *A. occidentale* leaves was incorporated in the preparation of gel formulation using carbopol 971 P NF polymer as a gelling agent. Three formulae 1, 2 and 3 of gel containing varying concentrations of carbopol 971 P NF (0.5, 1 and 1.5%) were prepared as shown in Table 1. Carbopol 971 P NF was soaked in distilled water overnight. The aqueous alcoholic extract of *A. occidentale* leaves was dissolved in the ethanol. The extract was then added to the soaked polymer. Propylene glycol was then added to the above mixture and stirred till a uniform suspension was obtained. After the addition was complete, gels were spontaneously formed with the addition of triethanolamine and adjustment of pH to 7. The prepared gel was referred to as F2-AAE-AOL.

Animals

The animals were procured from Bharat Serum and Vaccines Pvt. Ltd., Thane and housed in the animal house of C.U. Shah College of Pharmacy. Animals were acclimatized to the experimental room for one week and conditioned at room temperature and natural photoperiods. Animals were provided with standard food pellets as basal diet and water *ad libitum*. The study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethical Committee (IAEC) of C.U. Shah College of Pharmacy, S.N.D.T University, Mumbai. (Reg. no. 39/1999/CPCSEA). Male and female albino rats weighing 180-250 g were used.

Primary skin irritation study

To evaluate the safety of the prepared topical gel, a skin irritation study was conducted on albino rats as per OECD guidelines No. 404²¹. The irritation index was calculated to assess the irritation potential of the prepared carbopol gel according to Draize test²². The study was carried out after the approval of IAEC with Protocol No. CUSCP/IAEC/11/2013.

In vivo Wound healing activity

Wound healing activity was evaluated using excision, incision and dead space wound models in

rats. The study was carried out after the approval of IAEC with Protocol No. CUSCP/IAEC/10/2013 for incision and excision wound models and CUSCP/IAEC/11/2016 for dead space wound model. Four groups of animals containing six each were used for each of the excision, incision and dead space wound models. The animals of Gr. I were treated with a topical application of carbopol gel of aqueous alcoholic extract of *A. occidentale* leaves (F2-AAE-AOL). The animals of Gr. II served as the reference standard and were treated with betadine ointment. The animals of Gr. III were considered as the vehicle control and treated with plain carbopol gel. The animals of Gr. IV were left untreated (Negative control).

Incision wound model

An incision of 6 cm was made through the skin and cutaneous muscles on the depilated back of the rat using a scalpel blade. The incision was then closed with interrupted sutures with stitches 1 cm apart using black surgical thread and a curved needle. F2-AAE-AOL, plain carbopol gel and betadine were applied to different groups of rats, once daily from day 0 to day 9 post-wounding. The sutures were then removed on the 8th post wounding day and the breaking strength of the 10th day old wound was measured by tensiometer²³.

Excision wound model

A full thickness of the excision wound of a circular area (approx. 500 mm²) and 2 mm depth was made on the shaved back of the rats. F2-AAE-AOL, plain carbopol gel and betadine were topically applied once a day, to different groups of rats, starting from day 0 till complete epithelialization. The rate of wound contraction was measured as a percentage reduction of wound sizes every 4 d interval. A progressive decrease in the wound size was monitored periodically using transparency paper and a marker, and the wound area was assessed graphically to monitor the percentage of wound closure, which indicates the formation of new epithelial tissue to cover the wound. A number of days required for falling of Escher without any residual raw wound were recorded to estimate the period of epithelialization²⁴.

Dead space wound model

A longitudinal paraventral incision was made through the skin on the depilated back of the rat using a sterile scalpel blade. Dead space wounds were inflicted by implanting two sterilized cotton pellets

(10 mg), one on either side of the lumbar region on the ventral surface of each rat. The parted skin was kept together and stitched and closed with interrupted sutures (black surgical thread and curved needle) 1cm apart²⁵. F2-AAE-AOL, plain Carbopol gel and betadine were applied to different groups of rats, once daily from day 0 to day 9 post-wounding. On the 10th post-wounding day, granulation tissues formed on cotton pellets were dissected carefully. The wet weight of the granulation tissue was noted.

The granulation tissues were dried at 60°C for 12 h, and dry weight was recorded. The dried tissues were then hydrolyzed with 6 N HCl and kept at 110°C for 24 h in sealed glass tubes. This acid hydrolysate was used for the estimation of hydroxyproline and hexosamine content.

Determination of Hydroxyproline content

The above acid hydrolysate, neutralized to pH 7 was then added to 1mL of 0.01M CuSO₄, 1mL of 2.5 N NaOH and 1mL of 6% H₂O₂. All the tubes were incubated at 80°C for 5 min and tubes are chilled in an ice water bath. Upon cooling, 4 mL of 3 N H₂SO₄ and 2 mL of 5 % p-dimethylaminobenzaldehyde were added. The samples were incubated at 70°C for 16 min and then cooled. The absorbance was measured at 540 nm using spectrophotometer. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline at the same time²⁶.

Determination of Hexosamine content

About 1 mL of the acid hydrolyzed fraction was added to 1 mL of acetylacetone reagent in 0.5 N Na₂CO₃ and heated in a boiling water bath for 15 min. After cooling, 5 mL of 95 % ethanol and 1 mL of Ehrlich's reagent were added to it. The reaction was allowed to stand for 30 min to complete. The purple red colour was developed and measured after 30 min at 530 nm against the blank. The hexosamine content of the samples was determined from the standard curve prepared with D (+) glucosamine hydrochloride²⁷.

In the dead space wound model, granulation tissues formed on cotton pellet on the 10th post-wounding day were dissected out carefully and were used for the estimation of hydroxyproline

Estimation of growth factor and cytokine

The effect of a formulation containing aqueous alcoholic extract of *A. occidentale* leaves (F2-AAE-AOL) on inflammatory cytokine such TNF- α and

growth factor TGF- β was studied in rats using enzyme-linked immunosorbent assay (ELISA) technique with ELISA kits on granulation tissue homogenate obtained on 10th post-wounding day from dead space wound model. The procedures for estimation of TNF- α and TGF- β in rat tissue homogenates were carried out according to the manufacturer's instructions. The optical density of the yellow color was determined on the Microplate reader and washer (Ark Diagnostics Pvt. Ltd.).

In silico molecular docking studies

Docking studies were carried out using Grid-based Ligand Docking with Energetics (GLIDE) version 7.1 of Maestro molecular modeling suite 2016-2, Schrödinger LLC, NY, 2016. The protein structure with PDB ID 1Q5K (GSK3 β receptor), was retrieved from Protein Data Bank (www.rcsb.com) and used for validation of the docking algorithm. Once the crystal structure was exported, all the water molecules were deleted; bond orders and charges of inhibitor were assigned properly in the protein preparation step. The centroid of the co-crystallized ligand was used as the centre for receptor grid generation for 1Q5K.

The structures of all compounds were constructed using a build panel in Maestro. Nitrofurazone, a reported inhibitor of the GSK3- β receptor²⁸ was used as a reference standard for the docking studies on GSK3 β receptor. A unique low-energy 3D structure was generated for each ligand, including nitrofurazone, with the help of Ligprep, where appropriate hydrogens were added to all structures and subsequently energy minimization was carried out using the OPLS-2005 force field with a constant dielectric of 1.0. To validate the docking algorithm, the structure of N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl) urea, a native ligand for 1Q5K, was drawn in the "Build" panel of Maestro, prepared using LigPrep module and docked using the flexible ligand docking procedure in GLIDE. This docked pose of the ligand when superimposed on the original ligand pose in the crystal structure, matched, validating the docking protocol. The designed derivatives were docked using Extra Precision (XP) mode.

Statistical analysis

Results were expressed as Mean \pm SEM (Standard Error of Mean). Inter comparison between the groups were made using One way ANOVA followed by the Bonferroni post-test to compare every each group with each other on GraphPad InStat 3 statistical software and statistical significance was determined.

Results and Discussion

The preliminary screening of the bioactive aqueous alcoholic extract of *Anacardium occidentale* leaves was based on the extractive value, total phenolic content and total flavonoid content.

Percent yield of prepared extracts

The percent yield of all prepared extracts of *A. occidentale* leaves was determined and mentioned in Table 2. The aqueous alcoholic extract of *A. occidentale* leaves (AAE-AOL) showed the highest percent yield (28.77%) followed by methanol extract (12.16%), chloroform extract (7.14%), water extract (5.60 %) and petroleum ether extract (1.89%). This indicates that aqueous alcohol and methanol extracts contain large amounts of polar to moderately polar components such as tannins, flavonoids, glycosides and polyphenolic components.

Preliminary phytochemical screening of extracts

Preliminary phytochemical screening was carried out for all the extracts petroleum ether (60-80°C), chloroform, methanol, aqueous alcohol (50:50) and water) for detection and confirmation of various plant constituents such as tannins, alkaloids, glycosides, carbohydrates, proteins and flavonoids. The results are shown in Table 3. Extract of *Anacardium occidentale* leaves (AOL) revealed the presence of alkaloids, carbohydrates, flavonoids, saponins,

proteins, tannins and phenolic compounds in methanol, water and aqueous alcohol extracts. The chloroform extract and petroleum ether extract of AOL revealed the presence of tannins and phenolic compounds.

Determination of Total phenolic content of extracts

The total phenolic content of all prepared extracts of *Anacardium occidentale* leaves is shown in Table 2. The aqueous alcohol extract of *Anacardium occidentale* leaves (AOL) was found to contain a significantly higher amount of total phenolics (449.38±0.50 mg GAE/g of extract) as compared to methanol extract (267.87±0.81 mg GAE/g of extract), chloroform extract (44.52±1.40 mg GAE/g of extract), water extract (143.64 mg GAE/g of extract), and petroleum ether extract (12.74±0.17 mg GAE/g of extract).

Determination of Total flavonoid content of extracts

The total flavonoid content of aqueous alcohol extract (442.40 ± 3.72 mg RE/g of extract) was higher than methanol extract (237.27 ± 2.56 mg/g of RE), water extract (105.50 ± 2.02mg RE/g of extract), chloroform extract (83.43 ± 1.82 mg RE/g of extract) and petroleum ether extract (50.40 ± 1.47 mg RE/g of extract) (Table 2). This indicates that aqueous alcohol can be an efficient solvent for the extraction of polyphenolic compounds from AOL. Hence, from the results of the preliminary screening of extracts, the aqueous alcohol extract of *Anacardium occidentale* leaves (AAE-AOL) with good extractive value, the highest phenolics and flavonoids content was selected and further evaluated for its *in vivo* wound healing activity.

Primary skin irritation study

Topical gel formulation containing aqueous alcoholic extract of *Anacardium occidentale* leaves showed no erythema or oedema on intact rat skin and did not cause any skin reaction after examining at 24, 48 and 72 h. According to Draize *et al.*²², compounds producing scores of 2 or less are considered negative (no skin irritation). Since the primary skin irritation index of the developed gels was calculated as 0.00, the developed topical formulation was found to be nonirritant and safe for topical application.

In vivo wound healing activity

In all three models studied, the developed formulation F2-AAE-AOL exhibited significant wound healing potency as compared to reference standard Betadine and the negative control.

Table 2 — Yield (%), TPC and TFC of extracts of *Anacardium occidentale* leaves

Extracts	Yield (% w/w)	TPC (mg GAE/ g of extract)	TFC (mg RE/ g of extract)
Petroleum ether (60-80°C)	1.89±0.23	12.74±0.18	50.40±1.47
Chloroform	7.14±0.52	44.52±1.41	83.43±1.82
Methanol	12.16±0.86	267.87±0.81	237.27±2.56
Water	5.60±0.12	148.33±3.08	105.50±2.02
Aqueous alcohol (50:50)	28.77±0.52	449.38±0.50	442.40±3.72

[n=3; Values are represented as Mean ± SD]

Table 3 — Preliminary phytochemical screening of extracts of *Anacardium occidentale* leaves

Test	PEE-AOL	CE-AOL	ME-AOL	WE-AOL	AAE-AOL
Alkaloids	-	-	+	+	+
Carbohydrates	-	-	+	+	+
Glycosides	-	-	-	-	-
Saponins	-	-	+	+	+
Phenolic compounds and tannins	+	+	+	+	+
Proteins and amino acids	-	-	+	+	+
Flavonoids	-	-	+	+	+
Fixed oils and fats	-	-	-	-	-
Sterols and terpenoids	-	-	-	-	-

[PEE-AOL, CE-AOL, ME-AOL, WE-AOL and AAE-AOL: Petroleum ether, Chloroform, Methanol, Water and Aqueous alcohol extract of *Anacardium occidentale* leaves, respectively]

Table 4 — Effect of gel formulation F2-AAE-AOL on epithelialization period, breaking strength, hydroxyproline and hexosamine content of wounds in rats

Treatment groups	Epithelialization period (days)	Breaking strength (g)	Hydroxyproline content (mg/g of tissue)	Hexosamine content (mg/g of tissue)
F2-AAE-AOL	18.17±1.34*	704.67±4.17***	30.59±2.60	106.32±7.45*
Positive control	16.67±0.96**	614.33±3.40***	35.32±0.99**	113.79±4.73**
Vehicle control	24.33±1.30	409.17±3.75	26.6±0.71	94.67±7.28
Negative control	25±0.81	406.50 ±5.89	24.31±0.98	78.61±7.15

[Values are expressed as Mean ± SEM (n=6), * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. negative control. (One-way ANOVA, followed by Bonferroni post test). F2-AAE-AOL-gel formulation containing aqueous alcoholic extract of *Anacardium occidentale* leaves]

Incision wound model

The important parameter assessed in the employment of incision wound is the measurement of skin breaking strength on the 10th post-wound day using a tensiometer. F2-AAE-AOL showed a statistically significant ($P < 0.001$) increase in the breaking strength of rats as compared to Betadine and other treatment groups (Table 4). Since incision wounds treated with F2-AAE-AOL showed greater breaking strength, it may be concluded that it not only increases collagen synthesis per cell but also aids in cross-linking of the protein. An increase in collagen synthesis increases the breaking strength of the wound which indirectly increases the wound healing activity of the formulation.

Excision wound model

The excision wound model was employed to assess the potency of the drug to promote wound healing in Trauma type of the wound which was assessed by the rate of wound contraction and a number of days required for complete epithelialization of the wound area. The effect of a formulation containing an aqueous alcoholic extract of *Anacardium occidentale* leaves (F2-AAE-AOL) on the percentage of wound contraction is shown in Fig. 1.

The rats treated with F2-AAE-AOL showed statistically significant ($P < 0.05$) reduction in wound area on day 4 as compared to the negative control group. The rats treated with F2-AAE-AOL showed a statistically significant ($P < 0.001$) increase in % wound contraction on day 20 as compared to the negative control group. There was no significant difference in % wound contraction of rats treated with F2-AAE-AOL and standard Betadine on day 16 indicating that F2-AAE-AOL was having equivalent wound healing activity as that of standard Betadine on day 16. The period of epithelialization was significantly shorter in rats treated with F2-AAE-AOL (18 days), and the standard Betadine group (16 days) while in the negative control animals, it was delayed up to day 25 (Table 4).

Table 5 — Effect of gel formulation F2-AAE-AOL on TGF- β and TNF- α level of granulation tissue in rats

Treatment groups	TGF β level (pg/g of tissue)	TNF α level (pg/g of tissue)
F2-AAE-AOL	3577.38±2.09***	469.79±7.24***
Positive control	5732.38±9.51*	269.23±10.05*
Vehicle control	1780.78±7.9	1156.98±6.96
Negative control	1410.11±9.30	1289.41±5.56

[Values are expressed as Mean ± SEM (n=6), * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ versus negative control. (One-way ANOVA, followed by Bonferroni post test). F2-AAE-AOL-gel formulation containing aqueous alcoholic extract of *Anacardium occidentale* leaves]

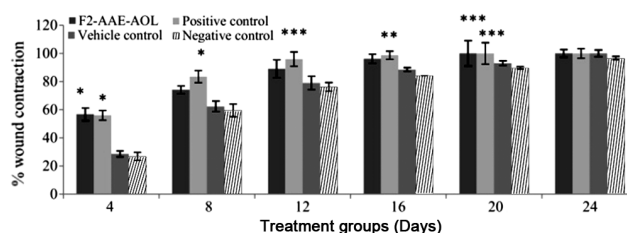


Fig. 1 — Effect of gel formulation F2-AAE-AOL on % wound contraction of excision wound in rats. [Values are expressed as Mean ± SEM (n=6), * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ vs. negative control. (One-way ANOVA, followed by Bonferroni post test). F2-AAE-AOL-gel formulation containing aqueous alcoholic extract of *Anacardium occidentale* leaves]

Dead space wound model

It is used to study the physical and mechanical changes in the granulation tissue. The biochemical markers, hydroxyproline and hexosamine, are evaluated in granulation tissue of dead space wounds to monitor the wound healing process. As shown in Table 4, F2-AAE-AOL treatment group exhibited higher hydroxyproline content in granulation tissues when compared with a negative control group and vehicle control group. F2-AAE-AOL showed a significant ($P < 0.05$) increase in hexosamine content as compared to a negative control group (Table 4).

Estimation of growth factor and cytokine

The effect of F2-AAE-AOL, standard Betadine and negative control group on growth factor TGF- β and inflammatory cytokines TNF- α is shown in Table 5.

On the 10th post-wounding day in the dead space wound model, a significant ($P < 0.001$) increase in the TGF- β concentration was observed in F2-AAE-AOL and standard Betadine treatment groups when compared with a negative control group. The data from ELISA analysis further showed a significant ($P < 0.001$) reduction in TNF- α level when rats were topically treated with F2-AAE-AOL (469.79 pg/gm of tissue) and standard Betadine (269.23 pg/g of tissue) as compared to negative control rats (1289.41 pg/g of tissue). The highest reduction in TNF- α level was observed in rats treated with standard Betadine as compared to a negative treatment group. The inhibition of the release of an inflammatory cytokine such as TNF- α in the wound site could therefore control the degree and duration of the inflammatory response contributing to successful wound closure

***In silico* molecular docking studies**

The known actives were subjected to molecular docking studies to understand through a computational approach, the molecular mechanism of wound healing effects of the developed formulation.

The crystal structures available in the protein data bank for GSK 3- β were 3Q3B, 3F7Z, 1H8F, etc. From these 21 available structures, 1Q5K had the highest resolution (1.94 Å) and it was the only protein crystal that has been co-crystallized with native ligand N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl) urea. Harish *et al.* also used 1 Q5K crystal structure for docking with GSK 3- β ¹⁸. Therefore, 1Q5K was selected for molecular modeling studies on GSK3- β .

Protein preparation

The Ramchandran plot of the fully prepared protein was analyzed and the protein was found to be minimized properly. The protein reports showed no steric clashes or missing atoms and minimum deviations for bond lengths, bond angles and dihedral angles.

Validation of docking protocol

An important consideration in docking studies is validation of the docking algorithm. Using a flexible docking procedure, the observed crystallographic structure was reproduced with a protein structure alignment score of 0.000, indicating that the docking protocol is valid and hence could be used for studying the interactions of the designed ligand (Fig. 2).

Docking studies of designed inhibitors

The docking studies were carried out for the known phytoconstituents to understand their interactions with

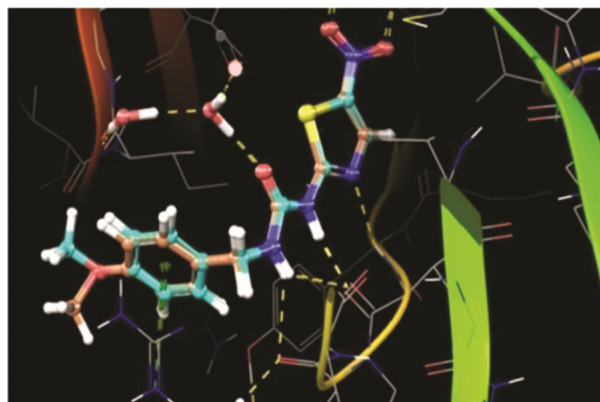


Fig. 2 — Comparison of native ligand and docked pose of N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl) urea.

Table 6 — Docking score of phytoconstituents with GSK 3 β (PDB:1Q5K)

Phytoconstituents	G score
Gallic Acid	-7.92
Ethyl gallate	-7.48
Methyl gallate	-7.48
Quercetin	-9.55
Nitrofurazone	-5.76

the receptor. The docking score of all the phytoconstituents on GSK3- β (PDB: 1Q5K) is shown in Table 6. The G-scores revealed that almost all the phytoconstituents such as gallic acid, methyl gallate, ethyl gallate and quercetin showed good binding affinity towards GSK3- β , compared to nitrofurazone, which is a standard drug used for wound healing. Binding interactions of methyl gallate, gallic acid, quercetin and nitrofurazone with GSK3- β (PDB 1Q5K) are shown in Fig. 3. The major hydrogen bond interaction of these ligands was seen to be with Valine 135. Quercetine showed additional π - π interaction with arginine 141 and tyrosine 134.

Delayed wound healing is one of the major therapeutic and economic issues in medicine today and has imposed a huge financial burden on both the developed and undeveloped world²⁹. Although there has been an enormous development in the pharmaceutical drug industry, wound healing drugs are not still satisfactory. Medicinal plants are in great demand due to a common belief that they are safe, reliable, clinically effective, low cost, globally competitive and better tolerated by patients. The plant based traditional therapy overcomes the problems associated with modern medicines such as high treatments cost, increased bacterial resistance and long manufacturing time¹¹. With this perspective in

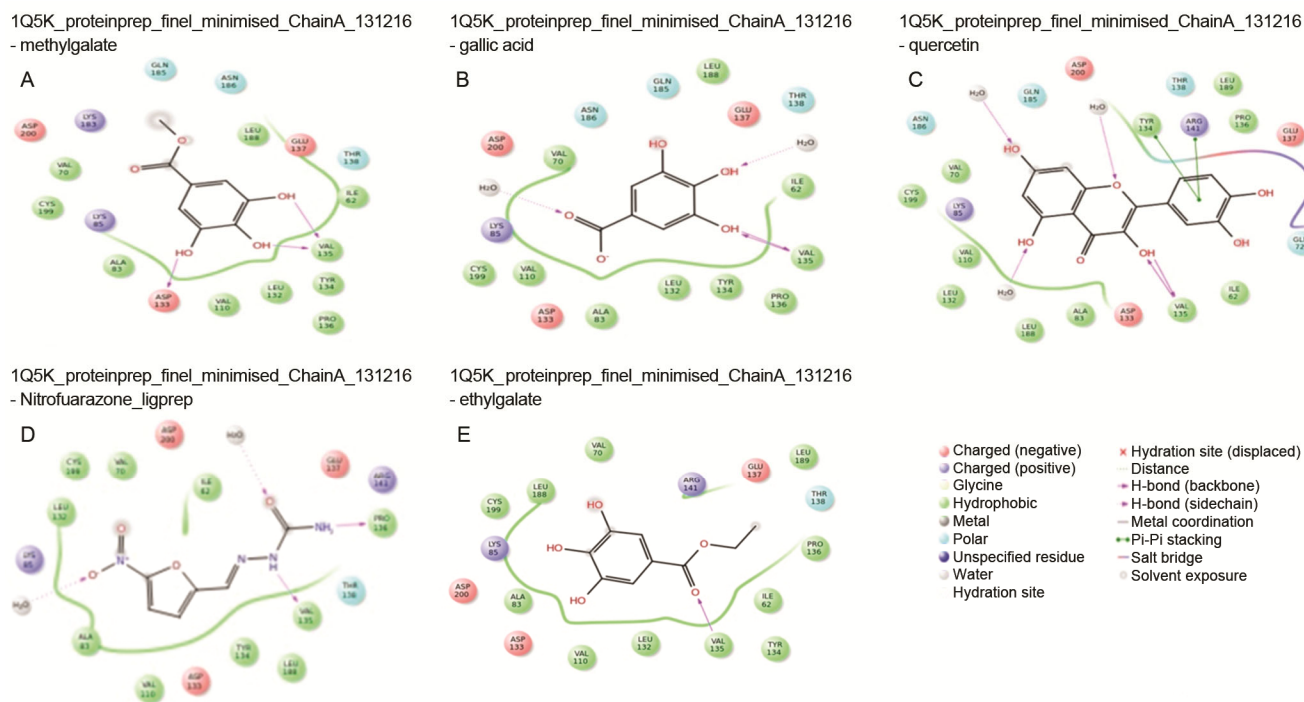


Fig. 3 — Interaction of GSK 3 β with (A) methyl gallate; (B) gallic acid; (C) quercetin; (D) nitrofurazone; and (E) ethyl gallate.

mind, the wound healing activity of gel of *Anacardium occidentale* leaves extract was evaluated by incision, excision and dead space wound models in rats.

In the present study, the aqueous alcohol extract of *A. occidentale* leaves showed the highest % yield as compared to methanol extract, chloroform, water and petroleum ether extract. This indicates that the aqueous alcohol extract of AOL contains large amounts of polar to moderately polar components such as tannins, flavonoids, glycosides and polyphenolic components. The phytochemical screening of extracts of *A. occidentalae* leaves (AOL) revealed the presence of alkaloids, carbohydrates, flavonoids, saponins, proteins, tannins and phenolic compounds in methanol, water and aqueous alcohol extracts. The chloroform extract and petroleum ether extract of AOL revealed the presence of tannins and phenolic compounds. Saponins and flavonoids are reported to possess wound healing activity. Plant metabolites such as polyphenols and flavonoids act as anti-inflammatory agents by preventing the synthesis of prostaglandins. Tannins have an active role in enhancing wound healing by improving regeneration and organization of the new tissue through their astringent and antioxidant properties³⁰. Therefore, the presence of these phytoconstituents in the crude extract may contribute to wound healing activity.

The excess free radicals generated in the inflammation phase of the wound healing process delay the healing process³¹. Hence, the therapeutic agent with free radical scavenging activity may help in accelerating the wound healing process and may prevent complications arising due to the delayed wound healing process. The polyphenols present in plants are responsible for the free radical scavenging effects. The activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers³². Hence, the present study is designed to determine the total phenolic content (TPC) and total flavonoid content (TFC) of the plant extracts and correlate them to their free radical scavenging activity.

The estimation of the phenolic content of extracts of *A. occidentalae* leaves was carried out using Folin-Ciocalteu reagent that produced blue colour by reducing yellow hetero polyphosphomolybdate-tungstate anions³³. The formation of an intense blue-coloured complex suggests the presence of a large number of hydrogen-donating groups in the phenolic compounds. The intense blue color of the aqueous alcohol extract of *A. occidentalae* leaves indicates higher content of total phenolic compounds as compared to other extracts. In the estimation of flavonoid content by the aluminium chloride

colorimetric method, the intense pink color produced due to the formation of the stable flavonoid-Al³⁺ complex indicates the higher flavonoid content³⁴. The AAE-AOL extract was found to contain higher total flavonoid content than other extracts. This indicates that the aqueous alcohol was found to be an efficient solvent for extracting total phenolics and flavonoids from AOL. Hence, an aqueous alcoholic extract with good extractive value and the highest phenolic and flavonoid content was selected for further *in vivo* wound healing activity.

The wound healing process involves the four phases viz. haemostasis, inflammation, proliferation or granulation, remodeling, or maturation. No single model is available to express the various components of the wound healing process³⁵. Hence, three different models (incision, excision and dead space wound models) were used to assess the effect of formulations containing plant extract on various phases of wound healing, which run concurrently but an independent of each other. Determination of various individual components of the phases of wound healing can provide important insights about events operative during the repair.

Significantly improved wound-healing activity was observed in all three models with a gel containing *A. occidentale* leaves extract when compared with that of the reference standard betadine and negative control (untreated wounds). All the parameters such as percent wound contraction, period of epithelialization, breaking strength, hydroxyproline and hexosamine content were affected with the treatment of a gel containing *A. occidentale* leaves extract.

The breaking strength is the strength of a healing wound and is measured experimentally by the amount of force required to disrupt it. In the incision wound study, the developed formulation F2-AAE-AOL showed a statistically significant ($P < 0.001$) increase in the breaking strength of incised wound when compared to negative control animals. This may be due to the increased synthesis of collagen and formation of stable intra- and intermolecular cross-linking³⁶.

The excision model is most widely used to evaluate the potential wound healing effects of substances. The parameters determined in the excision wound model are the wound contraction rate and reepithelialization of the skin. This open wound heals with re-

epithelialization, dermal reconstitution and contraction³⁷. Since this model is very similar to the clinical types of wounds, it is useful in investigating the healing process³⁸. In the excision model, gel formulation containing F2-AAE-AOL demonstrated wound healing activity as evidenced by an increase in the contraction rate and consequently a shorter period of reepithelialization. The standard betadine and F2-AAE-AOL showed a significant increase in wound contraction when compared to a negative control group. Contraction decreases healing time because it decreases the size of the wound and reduces the amount of extracellular matrix needed to repair the defect. The epithelialization time was also found to be significantly shorter in animals treated with standard Betadine and F2-AAE-AOL when compared to a negative control group. Epithelialization involves the migration and proliferation of epithelial cells across the wound area³⁹. Therefore, the shorter epithelialization time in the standard betadine and F2-AAE-AOL might be due to the facilitated proliferation of epithelial cells and/or increasing the viability of epithelial cells. The enhanced rate of wound contraction might be due to enhanced epithelialization in a shorter time. Thus, shorter epithelialization periods in extract-treated groups indicate that F2-AAE-AOL has a potential application as a wound healing agent.

The dead space wound model provides to assessment of wound collagen accumulation by measuring hydroxyproline content in granulation tissue. Collagen is a constituent of growing cells in healing tissues, which can be measured by monitoring the concentration of hydroxyproline⁴⁰. Thus, a higher concentration of hydroxyproline indicates a faster rate of wound healing. Rats treated with F2-AAE-AOL and standard Betadine showed an increase in hydroxyproline content of granulation tissue when compared to a negative control. The increased hydroxyproline content was found to indicate faster collagen turnover leading to rapid healing with a concurrent increase in the tensile strength of the treated wounds. Higher concentration of hydroxylproline found in the granulation tissues may be due to increased cellular proliferation and enhanced collagen maturation by increased cross-linking of collagen fibers, which indicating a faster rate of wound healing⁴¹.

The second measured important matrix molecule is hexosamine which acts as a ground substratum for the synthesis of new extracellular matrix and is secreted

during tissue repair. It was observed that rats treated with the developed formulation and standard betadine showed an increase in hexosamine content when compared to a negative control group. Hexosamine level increased in the early phases of wound healing indicates that fibroblasts are actively synthesizing ground material such as mucopolysaccharides on which collagen may be deposited⁸. Increased hexosamine level also reflects the stabilization of collagen molecules via enhanced electrostatic and ionic interactions; thereby augmenting wound healing⁴¹.

The regulation of cytokines and growth factors release, including TNF- α and TGF- β in the first stages of healing influences various processes at the wound site, such as stimulation of keratinocyte and fibroblast proliferation⁴², synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis, and regulation of the immune response⁴³. Rats treated with F2-AAE-AOL showed a significant decrease in TNF- α levels when compared with a negative control group. The inhibition of the release of an inflammatory cytokine such as TNF- α in the wound site could therefore control the degree and duration of the inflammatory response contributing to successful wound closure.

There was a significant increase in TGF- β level in granulation tissues of rats treated with F2-AAE-AOL when compared with a negative control group. This supports action in the pro-angiogenic phase of healing, increase fibroblasts proliferation and differentiation into myofibroblasts contributing to the formation of well organized granulation tissue and thereby, increasing wound contraction.

In silico molecular docking study was used to determine the binding energies of phytoconstituents in AAE-AOL extract with GSK3- β protein. In the docking study with GSK3- β (PDB ID:1Q5K), all the selected ligand molecules showed an encouraging docking scores. Quercetin revealed a maximum docking score, followed by catechin, methyl gallate, ethyl gallate and gallic acid and thus these are considered good inhibitors of GSK3- β receptor. Inhibition of the GSK3- β by the phytoconstituents present in AAE-AOL extract may contribute to their wound healing activity.

Conclusion

The present study demonstrated, possibly for the first time, the promising wound healing activity of a

developed topical formulation comprising the aqueous alcoholic extract of *Anacardium occidentale* leaves in rats. Our findings were supported by increased wound contraction, breaking strength, hydroxyproline and hexosamine content, TGF- β level and decreased level of TNF- α . The higher content of TPC and TFC found in the aqueous alcohol extract of *A. occidentale* leaves contributed to its antioxidant activity and thereby, accelerating the wound healing process. The probable mechanism of action might be due to the effective docking of the phytoconstituents present in bioactive extract against selected target GSK-3 β . F2-AAE-AOL enhances the wound healing process by preventing an over-expression of inflammatory response in the early stages of healing by mediating the production of pro-inflammatory cytokine and growth factor such as TNF- α and TGF- β . Hence, it can be concluded that a formulation containing an aqueous alcoholic extract of *Anacardium occidentale* leaves may have potential value as a topical wound healing agent to accelerate wound healing.

Conflict of interest

Authors declare no competing interests.

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