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Original Article

EVALUATION OF SAFETY PROFILES OF ANURADHA OIL-AN HERBAL WOUND HEALING FORMULATION IN LABORATORY ANIMALS

PATANKAR SB[1], MUJUMDAR AM[1], SANE RT[2], REMYA U[2]

[1]ACE Hospital & Research Centre, 32/2a, Erandwana. Pune 411004, [2]Department of Chemistry, Guru Nanak Institute of Research and Development, Matunga. Mumbai 400019
Email: researchamai@gmail.com

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ABSTRACT

Objective: To investigate Acute, Sub-acute dermal toxicity, Mucus membrane irritation activity of Anuradha Oil's (AO and AO11) – An herbal formulation having wound healing potential.

Methods: The formulations were derived from *Curcuma longa* Linn and *Glycyrrhiza glabra* Linn rhizhomes, *Hamiltonia suaveolens* Roxb bark, *Typha angustifolia* Linn flowers, *Azadirachta indica* A. Juss leaves without/with pig fat (AO and AO11 respectively)mixed in certain proportion using *Sesamum indicum* Linn oil as a base, manufactured at a GMP certified facility. Acute, sub-acute dermal safety, mucus membrane irritation profiles were studied by OECD guidelines number 434, 410 and 405 in Wistar rats and New Zealand rabbits respectively. For acute study dermal application of 2000 mg/kg single dose and for sub-acute study limit dose protocol of 1000 mg/kg for 28 days was used. For mucus membrane irritation test 0.10 ml dose was used.

Results: In an acute study no local reaction, behavioral changes and mortality was observed at 2000 mg/kg by dermal route till 14 days. In sub-acute study with dermal application of 1000 mg/kg for 28 days, there were no abnormal signs/symptoms at the application site, no difference in body weight, food consumption, hematology/enzyme profiles, urine analysis, relative organ weights and histopathological observations of vital organs in comparison to control animals. Mucus membrane of rabbits showed no evidence of irritation for both oils.

Conclusion: Acute dermal LD50 cut off for AO and AO11was found to be>2000 mg/kg, safe for dermal application for 28 days using the dose of 1000 mg/kg and non-irritant.

Keywords: Anuradha oil, Dermal toxicity, Acute/Sub acute safety, Mucus membrane irritation, Rats/ Rabbits.

INTRODUCTION

Wound healing is a complex and dynamic process sets in response to injury, to restore cellular, structural as well as functional integrity of injured tissue layers [1]. This biological process begins with trauma and ends with scar like grazes, burns, surgical incisions, stabs, leg ulcers, diabetic foot, decubitus ulcers (pressure sores), etc. It is generally divided into four distinct but overlapping phases viz. Hemostasis, inflammation, proliferation and remodelling [2]. Presently, there is no treatment of choice for wound healing due to complex physiological process; however, honey, silver nano-particles, copper complexes, stem cells, broad spectrum antibiotics, etc are used. Newer techniques like VAC (Vacuum-Assisted-Closure) and Hyperbaric Oxygen Therapy (HBOT) have been attempted for wound healing with encouraging results but are costly and having limitations [3]. Simultaneously, there are multiple herbal formulations available in the market; the majority of them are derived from different local herbs with limited scientific data. In Ayurvedic literature variety of herbs are recommended for the wound healing purpose [4]. However, the scientific data for understanding the underlying mechanism of action is rarely available. As per reverse pharmacology concept, for formulations based on traditional knowledge safety remains the most important starting point and the efficacy becomes a matter of validation [5]. We have developed two types of herbal formulations "Anuradha oil without pig fat" (AO) and "Anuradha oil with pig fat" (AO11) based on Ayurvedic literature, methodology and personal experience for the wound healing purpose. In the present communication results of Acute, Sub acute dermal safety and Mucus membrane irritation profile of AO and AO11 in laboratory animals are reported.

MATERIALS AND METHODS

materials

Preparation of AO & AO11

Rhizhomes of *Curcuma longa* Linn (*Haldi*) and *Glycyrrhiza glabra* Linn (*Jesthamadh*), bark of Hamiltonia *suaveolens* Roxb (*Jeetsaya*), flowers of Typha *angustifolia Linn* (*Ramban*), Azadirachta *indica* A. Juss (*Neem)* leaves were procured and authenticated at Agharkar Research Institute, Pune. The water extracts of the above materials with/ without pig fat [6] were used in certain proportions with *Sesamum indicum* Linn oil as a base at a GMP certified facility; Vishvaranga Ayurved Pharmacy, Pune for the manufacturing of these formulations. Standards of AO & AO11 and its chromatography profiles were investigated at Guru Nanak Institute for Research and Development, Mumbai.

Animals

Wistar rats of both sex and female New Zealand rabbits weighing between 200-300 g and 1.55-1.85 kg respectively were used for the proposed studies. They were housed in the CPCSEA approved animal house facility of the National Toxicological Centre, Pune (NTC). They were housed in polypropylene cages in an air-conditioned area at 22±2 ^{0}C and 12 hours light and dark cycle. They were provided with pellets of balanced animal food of Nav Maharashtra Chakan Oil Mills, Pune and water was provided *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee of the NTC, Pune.

Methods

Acute dermal toxicity study

Wistar rats of either sex were employed using OECD guideline number 434 [7]. Prior to application of the drug under study, the fur on the dorsal area of the trunk was removed with the help of electric clippers, exposing about 10% of the total body surface area. Based on exploratory studies, 2000 mg/kg doses were selected for the final study in 5 male and female rats respectively. AO & AO11 were applied to shaved area and secured with gauze and adhesive tape for 24 hours. Initially, animals were observed for four hours and subsequently after 24 hours twice a day for 14 days for any local changes, behavioral changes and mortality if any. At the end of the study, necropsy observations of all animals were recorded.

Sub-acute dermal toxicity study

This study was conducted using 10 males and 10 females Wistar rats employing OECD guideline number 410 [8]. They were divided into 2 equal groups viz., control and test. Prior to application of the study drug, the fur on the dorsal area of the trunk was removed with the help of electric clippers. Based on exploratory studies, the limit dose of 1000 mg/kg was applied once daily on the shaved area for 28 days. Initially, all the animals were observed continuously for four hours and monitored daily at least twice for the oil application site, behavioral changes, health status, signs of any abnormalities and morbid condition or death if any. The body weight and food intake of the rats was determined once every week. At the end of the experimental period blood samples were withdrawn from the retro-orbital plexus. Blood and serum samples were used for various hematological and biochemical parameters. The hematological parameters were Hb, PCV, Erythrocytes, Leucocytes count total as well as differential by a fully automated blood cell counter ERMA PCE-210. The serum was separated from the blood by centrifugation and stored at – 20°C for analysis of biochemical parameters viz. Glucose, SGPT, SGOT, BUN, ALP and total Proteins using a semi automatic clinical chemistry analyzer AGD 400. The rats were mildly anaesthetized under Pentobarbitone and sacrificed by cervical dislocation for necropsy observations and then the weights of vital organs like brain, liver, kidneys, lungs, spleen, adrenals, heart and ovaries/testis were determined. They were expressed as relative % weight in g. Liver, kidney and skin patches were preserved in 10% formalin solution. Subsequently, they were embedded in paraffin. They were sectioned and stained with hematoxylin and eosin to examine under the microscope for histological observations.

Mucus membrane irritation test

This study was conducted in 3 female New Zealand rabbits employing OECD guideline number 405 [9]. The test material was applied to vaginal mucosa of rabbits in a dose of 0.10 ml. The animals were observed for irritation score initially for 4 hours and subsequently once in 24 hours till 7 days to determine reversibility and irreversibility if any.

Statistical analysis

All the results were expressed as Mean±S.D. The statistical analysis was carried out by using Prism card software. The treatment group animals were compared with control using one way analysis of variance (ANOVA) and results were expressed as statistically significant for value $P<0.05$.

RESULTS

Acute dermal toxicity study

In the acute dermal toxicity study all the experimental animals well tolerated, the dose of 2000 mg/kg of AO & AO11 by the dermal route. The site of application of AO & AO11 appeared normal and the animals showed no abnormal clinical signs /symptoms and necropsy observations in comparison to control animals, without any mortality till 14 days. Thus, the LD50 cut off for AO and AO11was found to be>2000 mg/kg body weight.

Mucus membrane irritation test

After a single application to mucus membrane of animals, it appeared normal with no itching, redness, flare and inflammation till the end of the study, thus the irritation score was found to be 0.00. Hence, AO and AO11was termed as non-irritant [10].

Sub-acute dermal toxicity study

In sub-acute dermal toxicity study, all rats appeared normal, moreover the site of application was comparable to control animals, showed no signs /symptoms of any abnormality at the limit dose of 1000 mg/kg by the dermal route after application of AO & AO11 for 28 days. In the present study, there was no significant change in body weight as well as food consumption in the male and female rats in the test group as compared to the control animals as shown in fig. 1, 2, 3 and 4.

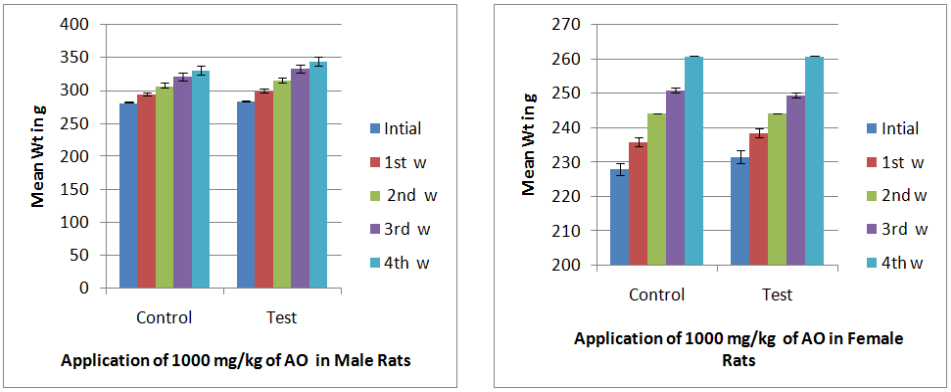

Fig. 1: Effect of dermal application of AO on mean body weights of rats

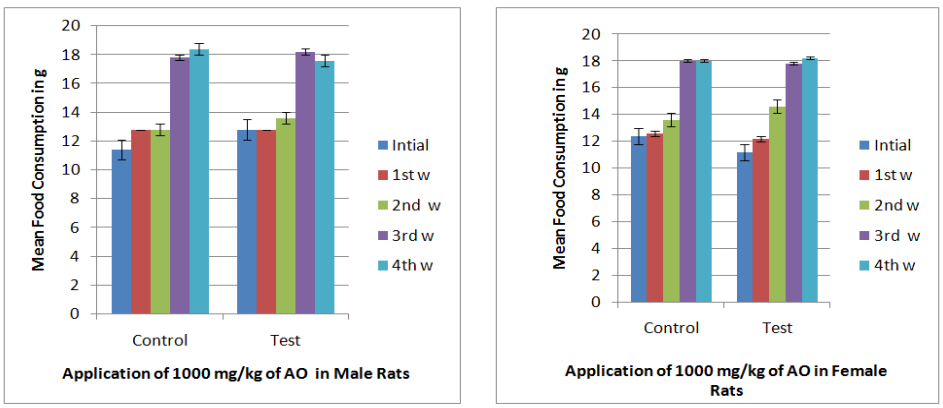

Fig. 2: Effect of dermal application of AO on mean food consumption by rats

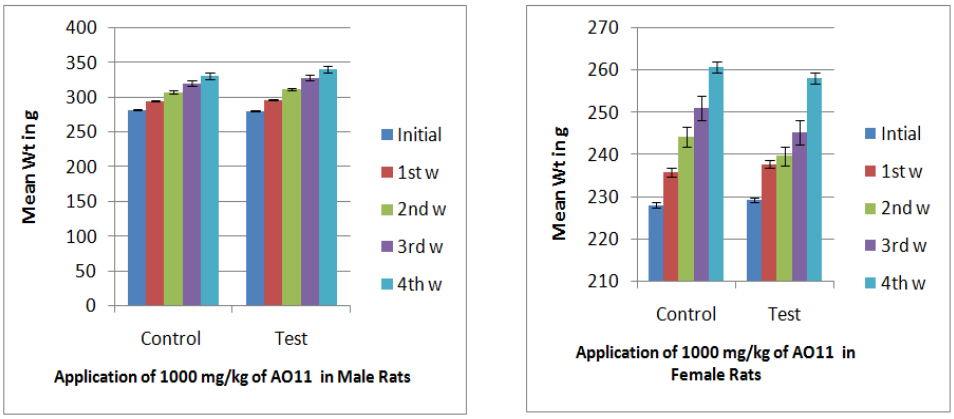

Fig. 3: Effect of dermal application of AO11 on mean body weights of rats

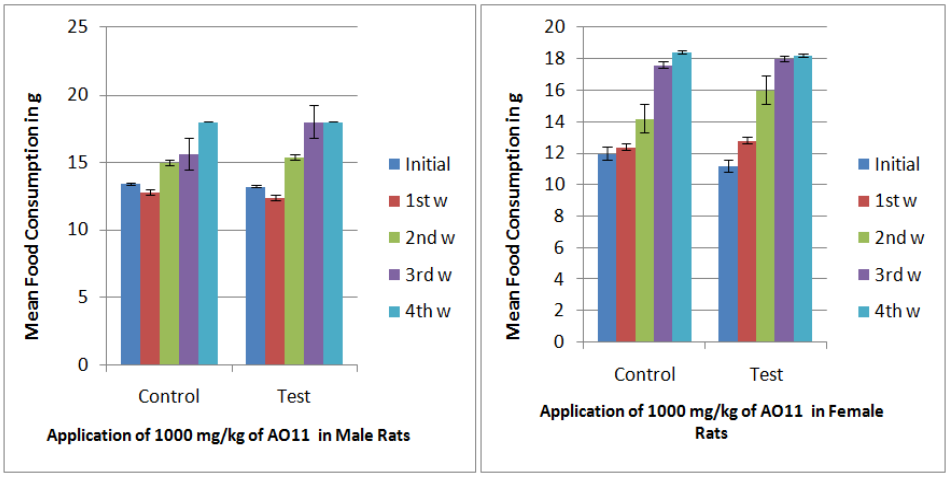

Fig. 4: Effect of dermal application of AO11 on mean food consumption by rats

After application of respective formulations in rats for 28 days, there was no statistically significant effect on different hematological parameters like Erythrocytes; Leucocytes count total and differential as well as Hb and significant effect on PCV and Th [12] in comparison to respective control animals as shown in Table1and 2. This indicates that both AO formulations are neither toxic to blood constituents, nor interfere with the production of their elements. Thus, AO formulations do not exhibit any toxic effect on Haemapoietic system. [11]

Table 1: Effect of AO dermal application on hematological parameters in Male / Female rats

Group	Hb (g/dl)	PCV %	Th (x $10^3/m^3$)	Erythrocytes (x $10^6/m^3$)	Leucocytes (x $10^3/m^3$)	N %	L %	E %	M %
Control (M)	12.30±0.99	28.80±3.16	494.00±10.51	4.040±0.527	5.762±1.425	37.60±3.88	57.80±4.02	1.0±0.0	3.56±0.29
Test (M)	12.50±1.52	35.58±14.87	677.20±9.524	3.960±1.180	5.962±2.501	32.80±6.69	63.30±6.82	1.0±0.0	2.94±0.17
Control (F)	12.20±0.80	33.80±6.38	469.40±6.841	7.640±5.223	7.200±1.434	30.90±8.29	64.80±8.46	1.0±0.0	3.30±0.20
Test (F)	13.90±1.57	45.60±4.53	629.20±10.83	11.14±3.411	8.384±0.852	35.10±5.02	61.00±4.79	1.0±0.0	2.96±0.28

Note: No difference observed in control and treatment groups.

In the present study various liver enzymes like SGPT, SGOT, ALP, total proteins and BUN were not significantly altered due to dose of 1000 mg/kg of both AO formulations as compared to the respective control group as shown in table 3 and 4. This indicates that there is no hepato or renal toxicity associated with the sub-chronic dermal exposure of AO and AO11 in rats [13].

Table 2: Effect of AO11 dermal application on hematological parameters in Male / Female rats

Group	Hb (g/dl)	PCV %	Th (x $10^3/m^3$)	Erythrocytes (x $10^6/m^3$)	Leucocytes (x $10^3/m^3$)	N %	L %	E %	M %
Control (M)	12.3±0.99	28.8±3.16	494.0±10.51	4.04±0.53	5.36±0.65	37.6±3.88	57.8±4.02	1.0±0.0	3.56±0.29
Test (M)	13.0±1.20	42.9±3.54	586.2±14.20	7.70±0.76	6.64±1.99	49.7±6.90	45.6±7.49	1.0±0.0	3.74±1.11
Control (F)	11.8±1.12	33.8±6.38	471.4±8.649	7.20±1.43	7.200±1.434	65.5±8.10	30.2±7.92	1.0±0.0	3.30±0.20
Test (F)	12.6±4.49	41.8±2.13	574.0±11.42	7.64±0.40	4.08±1.01	67.5±8.04	28.5±7.96	1.0±0.0	2.98±0.46

Note: No difference observed in control and treatment groups.

Table 3: Effect of AO treatment on blood chemistry parameters in Male/ Female rats

Group	Plasma glucose mg%	BUN mg%	Total proteins g%	SGOT U/l	SGPT U/l	ALP U/l
Control (M)	102.40±12.42	34.03±4.839	6.160±0.635	153.7±17.94	55.66±13.00	358.40±70.71
Test (M)	97.20±11.20	33.05±8.507	6.880±0.585	117.4±32.60	47.55±7.164	271.80±31.32
Control (F)	97.40±9.813	32.86±6.378	6.900±0.903	142.40±17.43	51.04±9.817	373.60±47.88
Test (F)	90.40±8.91	32.71±2.834	6.260±0.493	157.30±19.19	62.41±7.645	356.80±52.80

Note: No statistical difference observed in control and treatment groups.

Table 4: Effect of AO11 treatment on blood chemistry parameters in Male/ Female rats

Group	Plasma Glucose mg%	BUN mg%	Total proteins g%	SGOT U/l	SGPT U/l	ALP U/l
Control (M)	102.4±12.42	34.03±4.839	6.16±0.63	153.7±17.94	55.66±13.00	358.40±70.71
Test (M)	101.8±10.55	30.44±6.619	6.52±0.59	148.1±14.08	56.53±7.973	362.0±27.15
Control (F)	97.40±9.813	32.86±6.378	6.90±0.90	142.4±17.43	51.04±9.817	373.6±47.9
Test (F)	95.80±10.83	36.10±7.244	7.12±0.90	115.9±42.47	41.21±5.732	388.0±38.61

Note: No statistical difference observed in control and treatment groups.

There was no statistical difference in the relative organ weight in the test group when compared to control group as shown in table 5 and 6.

Table 5: Mean % relative organ weight after AO Treatment in Male/Female rats

Group	Adrenals	Heart	Kidneys	Liver	Spleen	Lungs	Brain	Testes/ ovaries
Control (M)	0.020±0.002	0.330±0.032	0.715±0.044	3.140±0.387	0.297±0.121	0.554±0.105	0.519±0.059	0.836±0.054
Test (M)	0.022±0.002	0.345±0.053	0.711±0.098	3.255±0.301	0.292±0.074	0.648±0.120	0.500±0.030	0.822±0.123
Control (F)	0.028±0.004	0.325±0.047	0.623±0.079	3.133±0.566	0.317±0.076	0.720±0.213	0.581±0.029	0.057±0.014
Test (F)	0.029±0.003	0.356±0.058	0.594±0.069	3.291±0.844	0.281±0.071	0.685±0.162	0.632±0.068	0.047±0.005

Note: No statistical difference observed in control and treatment groups.

Table 6: Mean % relative organ weight after AO11 Treatment in Male/Female rats

Group	Adrenals	Heart	Kidneys	Liver	Spleen	Lungs	Brain	Testes/ ovaries
Control (M)	0.0203±0.0018	0.330±0.032	0.715±0.044	3.140±0.387	0.297±0.121	0.554±0.105	0.519±0.059	0.836±0.054
Test (M)	0.0215±0.0040	0.371±0.034	0.761±0.038	3.554±0.376	0.312±0.087	0.650±0.041	0.531±0.041	0.602±0.155
Control (F)	0.0282±0.0045	0.325±0.047	0.623±0.079	3.133±0.566	0.317±0.076	0.720±0.213	0.581±0.029	0.057±0.014
Test (F)	0.0288±0.0053	0.331±0.013	0.636±0.061	3.416±0.386	0.382±0.123	0.772±0.188	0.624±0.085	0.060±0.009

Note: No statistical difference observed in control and treatment groups.

DISCUSSION

Acute dermal toxicity study revealed that both the oil formulations (AO & AO11) are non-toxic and according to the Global Harmonized Classification System (GHS) the LD50 cut off for AO & AO11 was found to be>2000 mg/kg body weight, which is GHS category-5 [7].

As in initial studies the LD50 cut off for both the formulations was>2000 mg/kg, limit dose of 1000mg/kg was considered for sub-acute dermal toxicity study. Alteration in the hematological parameters in Sub-acute dermal toxicity indicates systemic absorption of test material. In the present studies after application of AO & AO11 in rats for 28 days, there was no stastitically significant effect on Erythrocytes; Leucocytes count total and differential as well as Hb but showed significant effect on PCVand Th in comparison to respective control animals as shown in Table1and 2. As per CPCSEA guidelines, if the values lie in specified range then the significance is to be considered as non-toxic [12]. Hence, this indicates that both the formulations are neither toxic to blood constituents, nor interfere with the production of their elements. Thus, AO & AO11 do not exhibit any toxic effect on Haemapoietic system. [11, 12] Moreover, systemic toxicity of study drug is carried out by changes in important markers of blood biochemistry. Alteration in blood enzymes indicates cellular/tissue injury leading to systemic leakage of them from intracellular sites or target tissues [10]. Various liver enzymes like SGPT, SGOT, ALP, total proteins and BUN were not significantly altered of AO as compare to the respective control group as shown above. This indicates that there is no hepato or renal toxicity associated with the sub-chronic dermal exposure of AO and AO11 in rats [13]. In addition the plasma glucose levels were unaltered after repeated administration of AO as compared to control group animals, indicating that AO did not influence carbohydrate metabolism or blood glucose regulation system.

Usually alteration in body weight is considered as an important parameter for the assessment of response of an individual to the drugs [14] and might also indicate its side effects [15]. Further, at the termination of the study usually absolute as well as relative weights of vital organs were determined. These are indicative of the changes resulted in functioning of various vital organs due to metabolic changes, secretion of enzymes/hormones leading to hyper/ hypoplasia and alteration in tissue architecture [16]. To support this at the termination of the study, the weights of vital organs like adrenals, heart, kidneys, liver, spleen, lungs, brain, testis/ovaries were determined, converted into relative % organ weight and were compared with control rats as shown in table 5 and 6. There was no significant difference in the relative weight of vital organs of rats from both groups, indicating no significant effect on vital organs due to dermal application of test material for 28 days.

In gross necropsy observations there was no difference in control and both AO treated groups. Moreover, there were no histo-pathological changes observed in the sections of liver and kidney. This is supported with observations recorded in blood chemistry parameters as mentioned earlier. In the skin section more collagen deposits were recorded in AO treated animals in comparison to control. Usually collagen is considered as the natural substrate for cell attachment as well as growth, proliferation and differentiation promoting faster wound healing [17]. Thus, in sub-acute dermal application of 1000mg/kg of AO and AO11 for 28 days in rats, there

was no significant effect on body weight, food consumption, hematological as well liver enzyme profiles, relative organ weight and histopathological observations of vital organs like liver and kidney in comparison to control animals.

CONCLUSION

Acute dermal LD50 cut off by employing OECD guidelines in Wistar rats for AO and AO11 was found to be>2000 mg/kg body weight after observing for 14 days. Both oils were found to be non –irritant in Mucus Membrane Irritation test in rabbits. In sub-acute dermal application with 1000 mg/kg for 28 days in rats, there were no local /behavioral changes, alteration in blood profiles, biochemistry parameters, vital organ profiles and mortality in comparison to control animals for AO and AO11.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Nguyen DT, Orgill DP, Murphy GF. The pathophysiologic basis for wound healing and cutaneous regeneration. In: Biomaterials for Treating Skin Loss. Cambridge/Boca Raton: Woodhead Publishing (UK/Europe) & CRC Press (US); 2009. p. 25-57.
2. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg 1998;176(Suppl 2A):26S–38S.
3. Kranke P, Bennett M, Roeckl-Wiedmann I, Debus S. Hyperbaric oxygen therapy for chronic wounds. In: Cochrane Database Syst Rev (2): Kranke, Peter, ed.; 2004.
4. Deshpande AP, Javalgekar RR, Ranade S. Dravyagun Vidhnyan. Vol. 1&2, Anmol Prakashan, Pune; 2007. p. 2.
5. Patwardhan B, Vaidya ADB. Natural products drug discovery: Accelerating the clinical candidate development using reverse pharmacology approaches. Indian J Exp Biol 2010;48:220-7.
6. Joshi YG. Charak Samhita, Vaidyamitra Prakshan. 1st Ed. 2003:1:86, 43.
7. Organization for Economic Cooperation and Development, OECD Guidelines for Acute Dermal Toxicity-Fixed Dose Procedure. Test guideline 434. Draft guideline. [Last accessed 14 May 2004].
8. Organization for Economic Cooperation and Development, OECD Guidelines for testing of chemicals, “Repeated dose dermal toxicity: 21/28 days Study”. Test guideline 410. [Last accessed 12 May 1981].
9. Organization for Economic Cooperation and Development, OECD Guidelines for testing of chemicals, Acute Eye Irritation/ Corrosion. Test guideline 405. [Last accessed 24 Feb 1987].
10. Draize J, Woodward G, Calvery O. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membrane. J Pharmacol Exp Ther 1944;82:377-90.
11. Organization for Economic Cooperation and Development, OECD Guidelines for the testing of chemicals, Test guideline 408, repeated Dose 90-day Oral Toxicity Study in Rodents; 1995.
12. CPCSEA guidelines for Laboratory Animal Facility. Annexure 1- Hematological Data of Commomly used Laboratory Animals.
13. Adeneye AA, Ajagbonna OP, Adeleke TI. Preliminary toxicity and phytochemical studies of the stem bark aqueous extract of Musanga cecropioides in rats. J Ethnopharmacol 2006;105:374-9.
14. Winder CV, Lembke LA, Stephens MD. Comparative bioassay of drugs in Adjuvant-induced arthritis in rats: Flufenamic acid, mefenamic acid and phenylbutazone. Arthritis Rheum 1969;12(Suppl 5):472-82.
15. Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V. A 90 day oral gavage toxicity studies of D-methyl penidate and DL methyl penidate in sprague dawley rats. Toxicol 2002;179(Suppl 3):183-96.
16. Dhumal R, Patil P, Selkar N, Chawda M, Vahlia M, Vanage G. Sub-chronic safety evaluation of Ayurvedic immunostimulant formulation Immuforte in rats in reverse pharmacology. Toxicol Int 2013;20(1):87–94.
17. Ruszczak Z. Effect of collagen matrices on dermal wound healing. Adv Drug Delivery Rev 2003;55(12):1595-611.