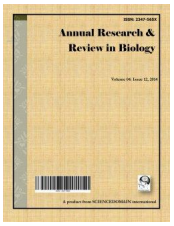


Annual Research & Review in Biology

18(3): 1-8, 2017; Article no.ARRB.35910
ISSN: 2347-565X, NLM ID: 101632869

Adult Zebrafish Model of Wound Inflammation to Study Wound Healing Potency of *Curcuma longa* Extracts

Sowmya Raghupathy[1], Lalitha Vaidyanathan[2*] and Lokeswari T. S. Sivaswamy[3]

[1]Department of Counselling Psychology, Madras School of Social Work, Egmore, Chennai, India.
[2]Department of Biomedical Sciences, Sri Ramachandra University, Porur, Chennai, India.
[3]Department of Biotechnology, Sri Ramachandra University, Porur, Chennai, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author LV designed the study, performed the statistical analysis and wrote the protocol. Author SR managed the literature searches, performed the study and wrote the first draft of the manuscript. Author LTSS guided and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2017/35910
Editor(s):
(1) Jean-Marie Exbrayat, Universite Catholique de Lyon, France.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Muhammad Shahzad Aslam, Universiti Malaysia Perlis, Malaysia.
(2) M. Angeles Esteban, University of Murcia, Spain.
(3) Sema Işisağ Üçüncü, Ege University, Turkey.
(4) Giani Maria Cavalcante, Institute of Technology of Pernambuco, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/21362

Original Research Article

Received 2nd August 2017
Accepted 4th October 2017
Published 12th October 2017

ABSTRACT

Aims: To understand the potential use of adult Zebrafish as a wound inflammation model to screen phytocompounds and chemically synthesized subjects.
Study Design: The experiment was designed to use healthy adult Zebrafish wound models, each group containing 30 fishes. One group was used as a control and others were the experimental groups. These were treated with the Curcuma longa extracts at various concentrations. The wound inflammation was studied in the treatment groups in terms of tissue regeneration and neutrophil migration.
Place and Duration of Study: Department of Biomedical Sciences, Sri Ramachandra University, during May 2015- August 2015.

**Corresponding author: E-mail: lalithav@sriramachandra.edu.in;*

Methodology: Caudal fin transection technique was used to establish the adult Zebrafish wound inflammation model. The study involved fin regeneration measurement and determination of neutrophil population at the wound site by performing histopathological staining of the tissue sections

Results: The fishes treated with aqueous extract had better wound healing compared to ethanol extract. The fishes treated with 500 µg of the aqueous extract showed maximum fin regeneration on day 5 compared to the control fishes. The same treatment also showed good neutrophil population at the wound site at 24 hrs post wounding and maximum resolution of inflammation after 24 hrs. The fishes treated with 500µg concentration of the aqueous extract showed twice the neutrophil population at 24 hrs and less than half of the neutrophil population at day 5pt compared with the untreated control group. The values were statistically analyzed using SPSS 17.0 version; for multivariate analysis Kruskal Wallis test was used. The values were highly significant at P<.01.

Conclusion: The study experimentally concludes the potential usage of adult Zebrafish as a model of wound inflammation to screen various bioactive fractions from plants or from synthetic origin for their wound healing abilities.

Keywords: Inflammation; Zebrafish; neutrophils; Curcuma longa; wound healing.

1. INTRODUCTION

Wound healing is a complex sequential process of restoration of the structural and functional properties of the damaged tissue. When unimpaired, wound healing progresses in a predictable fashion of three major phases: inflammation, proliferation and remodelling. After hemostasis, inflammation is the first cellular manifestation in a wound that occurs immediately, within a few hours after injury. The neutrophils recruited to wound site from the vascular network perform various effector functions like degranulation, phagocytosis, secretion of proinflammatory cytokines, and production of reactive oxygen species etc [1]. The neutrophil population at the wound site peaks at 24 hrs and declines gradually within a few days. Sustained inflammation makes the extracellular matrix to collapse and form necrotic centers [2]. Unresolved inflammation due to deregulation of immune system is the reason for delayed or impaired wound healing in case of aged and diabetic individuals [3,4].

Both the onset and resolution of inflammation is taken care of by the wound macrophages, through their role in release of cytokines that promote inflammatory response and inducing and clearing apoptotic cells respectively [3]. The wound inflammation is withdrawn through apoptotic clearance of the neutrophil population which initiates the actual healing process. A subset of this population gets repelled from the inflamed area and takes up reverse transendothelial migration to enter the vascular lumen as evident from the reverse migration studies in Zebrafish embryos [5]. Both the rate of neutrophil infiltration, thereby onset of inflammation, and the rate of resolution of inflammation can thus be used together as a gold standard parameter to assess wound healing potential of a substance. Currently, a wide range of animal models are available to evaluate wound healing potential, but the regular histopathological analyses in such evaluation processes check only the occurrence of inflammation qualitatively [2]. The rate of inflammation and its resolution is less reported in studies using animal models.

The study brings out a less expensive, more convenient and fast way of histopathological analysis to quantitate the wound healing potential of the extracts of the traditional medicinal plant, *Curcuma longa*, in terms of inflammation in experimental adult Zebrafish wound model. This would be a good alternative to mammalian wound inflammation models as the inflammation profile upon injury by tail transection in Zebrafish is comparable with that of the mammalian systems [6] and gives quick reproducible results. Though the mechanisms underlying skin physiology and function have been extensively studied, that behind the healing process has not been completely explored due to the lack of proper model systems. The present study would be a good contribution in establishing adult Zebrafish as a complete model system to study mammalian wound healing.

2. MATERIALS AND METHODS

Tricaine (MS-222) was purchased from Sigma. All the other chemicals and solvents used in the study were purchased from Sisco Research

Laboratories and were of highest purity grade [7,8].

2.1 Preparation of the Plant Extract

Dried rhizome of Curcuma longa (turmeric) were powdered and extracted with ethanol and water. 50 g of the dried powder was extracted with 200 ml of ethanol and kept in a shaker for 3 days at 160 rpm. Aqueous extract was prepared by the same procedure. The extract was concentrated by evaporation and the residue was stored in a sterile container till use.

2.2 Phytochemical Screening

The extracts were qualitatively screened for various phytochemicals according to the standard methods of plant analysis and the results were recorded [9].

2.3 Preparation of the Drug Formulation

The extract was mixed with 3% petroleum jelly to obtain a gel formulation for topical application [10]. The formulation was freshly prepared for each set of experiment.

2.4 Preparation of the Wound Model

The healthy adult Zebrafishes were purchased from Kolathur, Chennai and were bred in our laboratory. The fishes were allowed to acclimatize to the laboratory conditions for two weeks before the experiment. Fishes weighing close to 0.4 g were selected for the experiment. The fishes were grouped into control and two experimental groups, each group having 30 fishes including triplicates. The fishes were anaesthetized with tricaine for three and a half minutes. The caudal fin was transected 5 mm from the posterior end with sterile scalpel. The fishes were photographed before and after transection procedure with a digital camera connected to a stereo zoom microscope [11].

2.5 Treatment of the Wound Model with the Drug Formulation

The fin transected fishes of the experimental groups were treated with various concentrations, 100-500 µg, of ethanol and aqueous extracts. The drug formulations containing the respective concentration of the extracts were carefully applied on to the transected fin with gloved hands under aseptic conditions. The treated fishes were transferred to the recovery tank, after which they were grouped in separate tanks labeled with the concentration treated. Triplicates were maintained all through the study. The control fishes were treated with the formulation base alone.

2.6 Determination of Wound Healing Activity

2.6.1 Fin regeneration

The fishes were observed on 3, 5 and 7 days post-wounding for fin regeneration. The fin growth was measured and the results were photographed and recorded.

2.6.2 Neutrophil migration

Caudal fins of the treated zebrafish wound models and the control fishes (untreated) were sectioned and analyzed for neutrophil population at 24 hrs and on day 5 of observation. The treated zebrafishes were euthanized in tricaine for 10 minutes (according to the protocol in the zfin database). The fishes were fixed in formalin solution, embedded in paraffin wax and were sectioned in a microtome. The sections were stained with haematoxylin & eosin stain and observed for neutrophil population.

3. RESULTS

3.1 Phytochemical Screening

The phytochemical screening of the plant extracts revealed the presence of various phytoconstituents like flavanoids, saponins, glycosides, phenols, alkaloids and terpenoids (Table 1).

3.2 Determination of Wound Healing Activity

3.2.1 Fin regeneration

The fin growth measurement on days 1, 3, 5 and 7 were compared with the control. The fishes treated with the aqueous extract showed better regeneration compared to those treated with the ethanol extract with the rate of regeneration increasing with concentration (Fig. 1). The fishes treated with 500 µg of the aqueous extract showed maximum fin regeneration on day 5 compared to the control fishes (Table 2).

Table 1. Qualitative screening of the plant extract revealing its phytoconstituent profile

S. no.	Phytoconstituents tested	Ethanol extract	Aqueous extract
1	Flavanoids	+	+
2	Saponins	+	-
3	Glycosides	+	+
4	Phenols	+	+
5	Alkaloids	+	-
6	Terpenoids	-	+

'+' indicates presence, '-' indicates absence

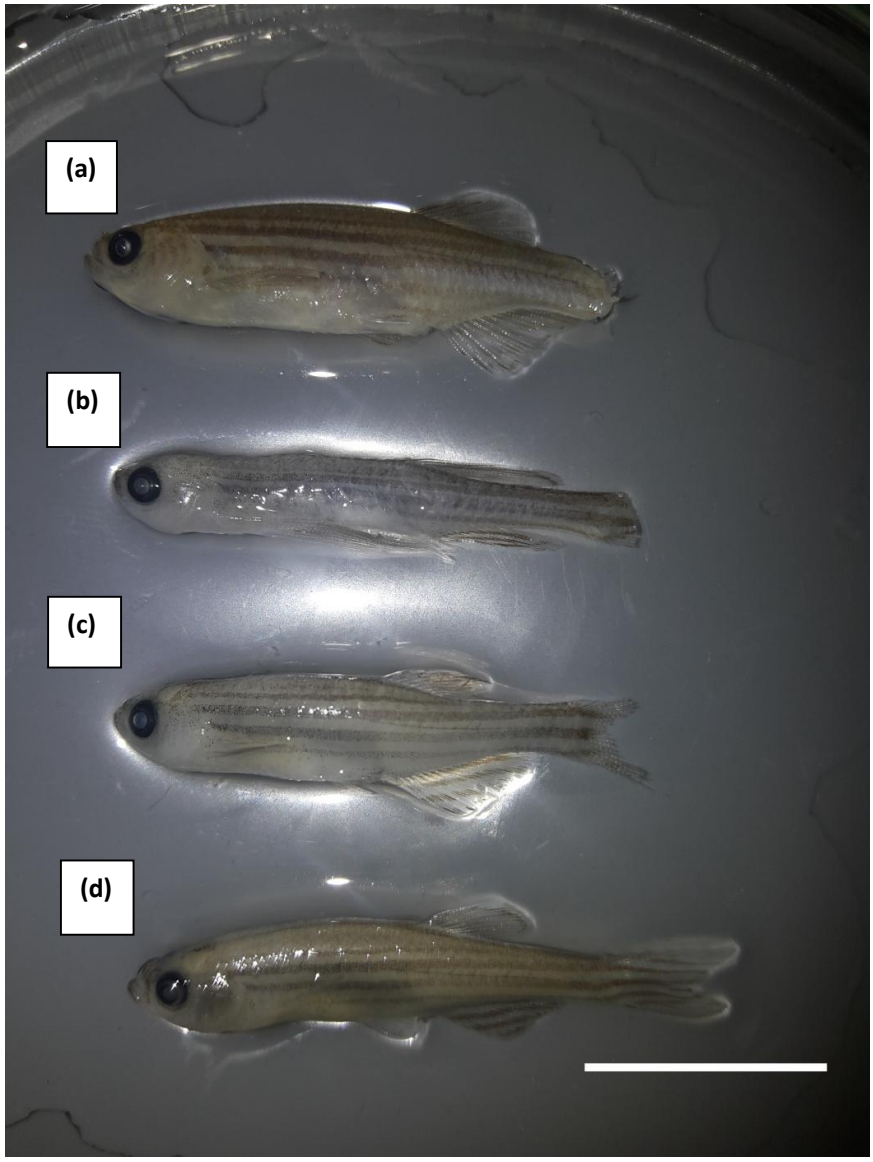
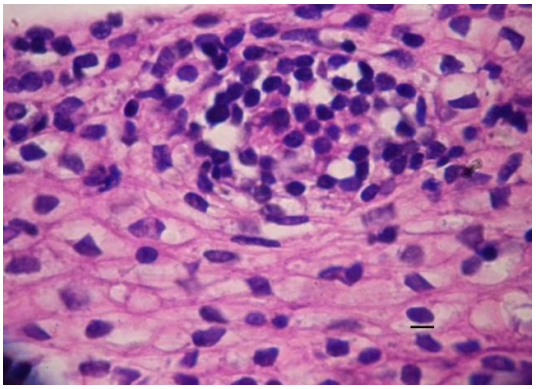
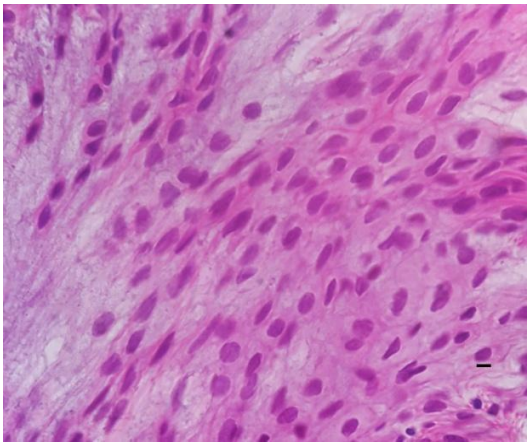


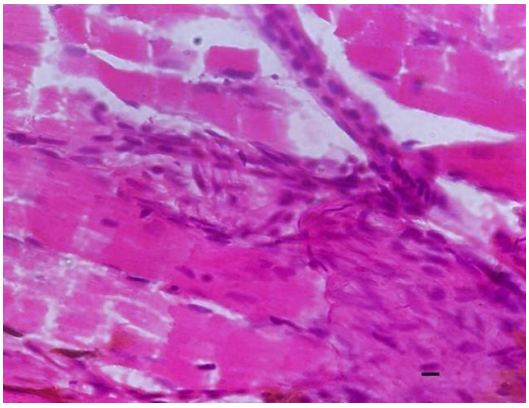
Fig. 1. Zebrafish caudal fin transection. (a) fish immediately after caudal fin transection, (b) control (untreated) fish on day 5 post-transection, (c) 500 µg ethanol extract treated fish on day 5 post-transection and (d) 500 µg aqueous extract treated fish on day 5 post-transection. Scale bar = 19 mm



(a)



(b)



(c)

Fig. 2. Neutrophil population (at 24 hrs) at the wound site of the fish treated with the aqueous extract (500 µg) (a), ethanol extract (500 µg) (b) and the control fishes (untreated) (c) analyzed using H&E staining. Scale bar = 30 µm

Table 2. Fin growth on day 5 post-transection in fishes treated with the aqueous and ethanolic extracts of the plant

Treatment	Concentration (µg)	Fin growth (Mean±S.D.) (mm)
Control	Formulation base	0.9±0.1
Ethanol extract	100	0.9±0.1
	200	1.2±0.3
	300	1.2±0.1
	400	1.3±0.2
	500	1.3±0.2
Aqueous extract	100	0.9±0.2
	200	1.2±0.1
	300	1.3±0.2
	400	1.4±0.1
	500	1.5±0.1

3.2.2 Neutrophil migration

The neutrophil population in the fin sections was found to peak at 24 hours and decrease in number on day 5 of observation (Fig. 2). The control fishes recruited less number of neutrophils compared to the ones treated with the extracts. Fig. 3 shows that compared to the fishes treated with ethanol extract, the fishes treated with the aqueous extract recruited more neutrophils at the wound site by 24 hours post wounding, the number increasing with concentration. On day 5, the neutrophil population drastically reduced in number compared to the control fishes which showed very less reduction in the number of neutrophils and hence theoretically less resolution of inflammation. Treatment with 500 µg of the aqueous extract was effective in resolution of inflammation compared to the ethanol extract group. Statistical analysis was done with SPSS 17.0 version to describe the data. For the multivariate analysis non-parametric Kruskal Wallis test was used. The values were expressed as mean ± S.D. for the determinations.

The results indicate that adult Zebrafish could be used as a successful alternative experimental model to reveal the potency of the plant extract in accelerating wound healing. The extract increases initial inflammation immediately after injury and regulates fast resolution of the same thereby healing the wound.

4. DISCUSSION

Zebrafish is a vertebrate capable of regeneration naturally. This regeneration property of the model

organism has been accelerated by treatment with *C. longa* aqueous extracts. This healing potency of the well known traditional plant could be attributed to the synergistic role of the various phytoconstituents in it. Phytochemical screening of the aqueous extract of the plant reveals the presence of flavanoids, glycosides, phenols and terpenoids. Polyphenolic compounds including flavanoids are said to promote wound healing through several cellular mechanisms, chelating the free radicals and reactive oxygen species, promoting wound contraction and increasing the formation of capillary vessels and fibroblasts. Their healing effect has been reported to be the consequence of suppression of acute inflammation due to time-dependant regulation of expression of the pro-inflammatory and repair-regulating chemokines [12]. Several polyphenol-collagen composites have been tested and proved effective for their potential usage in wound dressings [13]. Several glycosides have been reported to contribute to the anti-inflammatory and wound healing activities of therapeutic preparations [14,15]. Wounds are commonly thought to be complicated by infection [16]. The anti-microbial properties of flavanoids, phenols and terpenoids could have played an essential role in avoiding prolonged inflammation by eliminating the microbial load at the wound site [17]. The extract and its phytoconstituents might target the wound macrophages by triggering the release of pro-inflammatory cytokines during the initial stage of inflammation or/and apoptotic signals during the later stages.

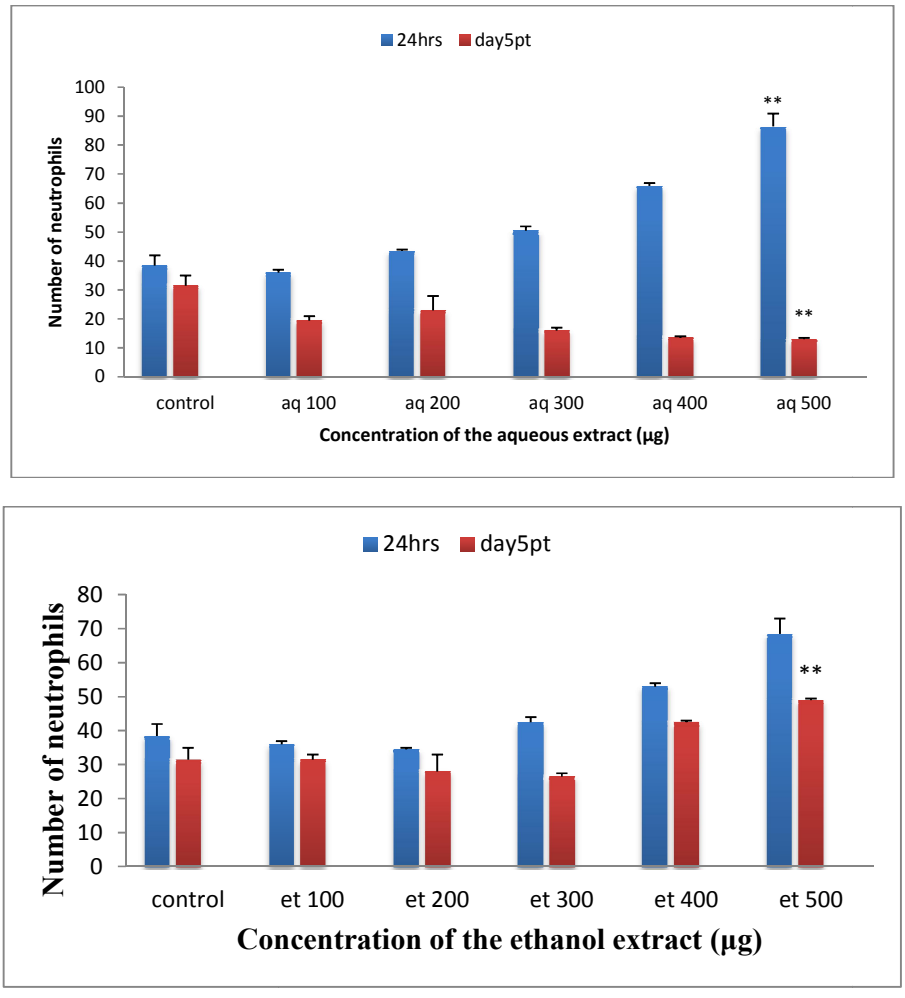


Fig. 3. Dose dependent onset and resolution of inflammation in wound models treated with different concentrations of the aqueous and ethanol extracts (100-500 µg) and the controls

***The values were highly significant at P<.01*

The molecular details are to be explored with advanced methods in the established model in the further studies. The plant extract tested thus exhibits its wound healing potency by regulating inflammation at the wound site through the above discussed mechanisms.

The adult Zebrafish fin regeneration system is a simple and excellent alternative model to reflect, both qualitatively and semi-quantitatively, the wound healing ability of molecules in terms of inflammation and regeneration, both of which are key events of the natural healing process.

5. CONCLUSION

The study experimentally concludes the potential usage of adult Zebrafish as a model of wound inflammation to screen various bioactive fractions from plants or from synthetic origin for their wound healing abilities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nathan C. Neutrophils and immunity: Challenges and opportunities. Nat Rev Immunol. 2006;6:173-82.
   DOI: 10.1038/nri1785
2. Wiksman LB, Solomonik I, Spira R, Tennenbaum T. Novel insights into wound healing sequence of events. Toxicol Pathol. 2007;35:767-79.
   DOI:https://doi.org/10.1080/01926230701584189
3. Guo S, DiPietro LA. Factors affecting wound healing. J Dent Res. 2010;89:219-29.
   DOI: 10.1177/0022034509359125
4. Nishio N, Okawa Y, Sakurai H, Isobe K. Neutrophil depletion delays wound repair in aged mice. Age. 2008;30:11-9.
   DOI: 10.1007/s11357-007-9043-y
5. Starnes TW, Huttenlocher A. Neutrophil reverse migration becomes transparent with Zebrafish. Adv Hematol. 2012;1-11.
6. Renshaw SA, Loynes CA, Trushell DMI, Elworthy S, Ingham PW, Whyte MKB. A transgenic zebrafish model of neutrophilic inflammation. Blood. 2006;108:3976-8.
   DOI: 10.1182/blood-2006-05-024075
7. Chiara U, Stefania N, Mauro G, Guido D, Simona F, Arnaldo C, Massimo A, Luca C, Marco P, Marco R. HIV-1 Tat and heparin sulphate proteoglycan interaction: A novel mechanism of lymphocyte adhesion and migration across the endothelium. Blood. 2009;114:3335-42.
8. Kathleen MC, Christa MW, Richard SB. A review of tricaine methanesulfonate for anesthesia of fish. Rev Fish Biol Fisheries. 2011;21:51-59.
   DOI: 10.1007/s11160-010-9188-0
9. Harborne JB. Phytochemical methods, a guide to modern techniques of plant analysis. Second Edition, Chapman and Hall; 1984.
10. Jaiswal S, Singh S, Singh B, Singh HN. Plants used for tissue healing of animals. Natural Product Radiance. 2004;3:284-92.
11. Jeanmarie MZ, Robert LT. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits Zebrafish caudal fin regeneration. Toxicological Sciences. 2003;76:151-161.
    DOI: https://doi.org/10.1093/toxsci/kfg205
12. Pastore S, Lulli D, Fidanza P, Potapovich A, Kostyuk VA, Luca CD, MikhalChik E, Korkina L. Plant polyphenols regulate chemokine expression and tissue repair in human keratinocytes through interaction with cytoplasmic and nuclear components of epidermal growth factor receptor system. Antioxid Redox Signal. 2012;16:314-28.
    DOI: 10.1089/ars.2011.4053
13. Gaspar A, Craciunescu O, Moldovan L, Ganea E. New composites collagen-polyphenols as potential dressing for wound care. Rom J Biochem. 2012;49: 173-81.
14. Korkina LG, Mikhalchik E, Suprun MV, Pastore S, Dal Toso R. Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally occurring biotechnologically produced phenylpropanoid glycosides. Cell Mol Biol. 2007;53:84-91.
    DOI: 10.1170/T822
15. Michael JR, Lisa C, Will W, Brian S, Paul M. Wound healing and inflammation: Embryos reveal the way to perfect repair. Phil. Trans. R. Soc. Lond. 2004;359:777-784.
    DOI: 10.1098/rstb.2004.1466

16. Akdemir Z, Kahraman C, Tatli L, Kupelli E, Suntar I, Keles H. Bioassay-guided isolation of anti-inflammatory, antinociceptive and wound healer glycosides from the flowers of *Verbascum mucronatum* Lam. J Ethnopharmacol. 2011;136:436-43.
DOI: 10.1016/j.jep.2010.05.059

17. Bharathidasan R, Mahalingam R, Ambikapathy V, Panneerselvam A. Screening of antibacterial activity and phytocompound studies of *Ochnandra travancorica* and *Bignonia unguicali*. Int J Pharm Sci Health Care. 2012;2:29-34.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21362