IJPSR (2012), Vol. 3, Issue 06 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 March, 2012; received in revised form 25 May, 2012; accepted 29 May, 2012

INVESTIGATION OF EFFECT OF *MURRAYA KOENIGII* ON BIOPHYSICAL AND BIOCHEMICAL PARAMETERS OF WOUND IN DIABETIC HYPERLIPIDEMIC WISTAR RATS

Vikram Kumar*[1], Angshu Bandyopadhyay [2] and Vikram Sharma [3]

CMJ University [1], Shillong, Meghalaya-793003, India
Sri Balaji College of Pharmacy [2], Jaipur, Rajasthan-302013 India
Department of Pharmacology, Sri Balaji College of Pharmacy [3], Jaipur, Rajasthan, India

Keywords:
Murraya koenigii (MK), Dead space wound, Ciprofloxacin, STZ-Streptozotocin, DH-Diabetic hyperlipedemic, hydroxyprolin

Correspondence to Author:

Vikram Kumar

Research Scholar, Faculty of Pharmacy, CMJ University, Shillong- 793 003, Meghalaya, India

ABSTRACT

Plants have been used in traditional medicine for several thousand years. India is perhaps the largest producer of medicinal herbs and is rightly called the "Botanical garden of the World". *Murraya koenigii* Linn. commonly known as Meethi neem, belongs to the family Rutaceae. The curry tree is native to India and it is found almost everywhere in the Indian subcontinent excluding the higher levels of Himalayas. The effect of *Murraya koenigii* leaves aqueous extract on biophysical and biochemical parameters of wound were studied by dead space wound model in diabetic hyperlipidemic rats. In dead space wound model, animals treated with *Murraya koenigii* (oral administration of variable dosage level 200mg/kg, 300mg/kg and 400mg/kg) leaves aqueous extract showed significant increase in Wet & Dry granulations tissue weight (biophysical parameter) and hydroxyprolin content (biochemical parameter) when compared to the diabetic hyperlipidemic control group rats. In this study, very significant ($p<0.001$) result was found with 300mg/kg dose level because the effect was dose dependent up to 300mg equivalent of extract. The results suggested that aqueous extract of *Murraya koenigii* possess significant wound healing potential in diabetic hyperlipidemic rats. Further studies may reveal the exact mechanisms of action responsible for the wound healing activity of *Murraya koenigii* leaves aqueous extract in diabetic hyperlipidemic condition.

INTRODUCTION: The function of skin is to serve as a protective barrier against the environment. Wounds are physical injuries that result in an opening or break of the skin.

After injury, the objective of wound healing is to restore structure and function to an injured tissue in order to approximate pre-wound characteristics. Healing is a complex and intricate process initiated in response to an injury that restores the function and integrity of damaged tissues.

Healing process can be broadly categorized into three stages; inflammatory phase (consisting the establishment of homeostasis and inflammation); proliferate phase (consisting of granulation, contraction and epithelialization) and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue [1].

The prevalence of chronic wounds in the community was reported as 4.5 per 1000 population whereas that of acute wounds was nearly doubled at 10.5 per 1000 population [2].

Healing of a chronic wound requires care that is patient centered, holistic, interdisciplinary and should be cost effective and evidence based. Several natural products [3], plant products, which are composed of active principles, like triterpenes and alkaloids and flavonoids [4] and biomolecules [5] have been reported to promote the process of wound healing.

Diabetic wounds are slow, non-healing wounds that can persist for weeks despite adequate and appropriate care. Such wounds are difficult and tough to manage [6]. Though the exact pathogenesis of poor wound healing in diabetic wounds is not clearly understood, evidence from studies involving both human and animal models reveal several abnormalities in the various phases of the wound healing process [7, 8].

Delayed and incomplete healing of wounds can be a major problem for diabetic patients. Macrophages are an important cell in the complex process of wound repair representing the major source of cytokines throughout the wound healing process. Cytokines mediate many of the cellular responses critical to timely wound repair. It has been suggested that diabetes impairs would healing through disruption of local cytokine production.

Previously it has been seen that platelet-derived growth factor B chain (PDGF-B) levels are deficient at the wound site of diabetic rats. The diabetic condition was associated with a generalized reduction of macrophage cytokine release.

Non-diabetic hyperlipidemic animals demonstrated similar cytokine reduction supporting the hypothesis that elevated serum lipids are the primary determinants of diabetes-induced reductions in macrophage cytokine release. Thus, manipulation of serum lipids may be a therapeutically useful modality for controlling macrophage cytokine release in the inflammatory and/or wound environment [9].

Murraya koenigii, belonging to the family Rutaceae, commonly known as curry-leaf tree, is a native of India, Srilanka and other south Asian countries. Leaves are rich in minerals, vitamin A, vitamin B, and are a rich source of carbohydrates, proteins, amino acids and alkaloids [10-11]. The plant has also been used in traditional Indian medicine systems for a variety of ailments [12-13].

It was found that reduction in total serum cholesterol and an increase in the HDL and lower release of lipoproteins into the circulation take place when rats were fed with a standard diet along with curry leaves [14]. Curry leaves also exhibited strong antioxidant property on liver and heart. It was found that phenolic antioxidant is present in *Murraya koenigii* and other herbs [15]. Hypoglycemic activity of *Murraya koenigii* on normal and diabetic rats was found [16]. The beneficial effects of *Murraya koenigii* leaves on antioxidant defense system and ultra structural changes of pancreatic beta cells in experimental diabetes in rats was studied [17]. There is no scientific report on wound healing effect of *Murraya koenigii* leaves extract on diabetic hyperlipidemic rats.

The present study has been undertaken to examine the wound healing potential of *Murraya koenigii* leaves aqueous extract in diabetic hyperlipidemic Wistar rats.

MATERIAL AND METHODS:

Freshly collected leaves of *Murraya koenigii* from medicinal garden of Sri Balaji College of Pharmacy, Jaipur, after authentication were shade dried and powdered to course powder size.

Extraction: The powder was extracted with distilled water using Soxhlet at boiling temperature (100^{o}C) up to 10 h. A dark brown color extract is obtained. This dark brown extract was cooled and filtered to remove the residue. The extract was concentrated on rotavapor under reduced pressure and then finally lyophilized to get a powder weighing about 75g [16].

Preliminary Phytochemical Studies: The extract was then subjected to qualitative phytochemical screening for the identification of the phytoconstituents [18-20].

Acute toxicity: The acute toxicity study was done by **"fixed dose"** method in healthy adult female albino Wistar rats according to CPCSEA recommended "OECD guidelines 420 [21].

Animal: Adult male and female albino Wistar Rats (250-300g) were obtained from animal house facility of Sri Balaji College of Pharmacy, Jaipur. Animal House Facility of this division is approved by Govt. of India, under the Ministry of Environment & Forest (Reg. No. 1212/ac/08/CPCSEA).

Then all the animals were acclimatized at least under standard husbandry condition i.e., room temperature 24±1^{o}C; relative humidity 45-55% and 12:12 hr light/dark cycle. The animal had free access to standard laboratory chow diet with water supplied *ad libitum* under strict hygienic condition. The rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anesthesia (10 mg/kg body weight of an animal).

Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study. The approval of the Institutional Animal Ethical Committee (IAEC) of Sri Balaji College of Pharmacy, Jaipur, was taken prior to start of experiments. All the protocol and experiment were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA).

Induction of Diabetic Hyperlipidemia (DH) [22-23]:

(Development of High Fat Diet-fed and Streptozotocin-treated type 2 diabetic rat's model): The rats were allocated into two dietary regimens normal pellet diet NPD and high fat diet HFD (2% cholesterol, 30% dalda and 68% of pellet chow) *ad libitum*, respectively, for the initial period of 2 weeks [22].

After the 2 weeks of dietary manipulation on the confirmation of hyperlipidemia in rats , a subset of the overnight fasted rats from each dietary group was injected intraperitoneally (i.p.) with low dose of STZ (35 mg/kg) while the respective control rats were given vehicle 0.1M citrate buffer (pH 4.5) in a dose volume of 1ml/kg, i.p., respectively [23].

The body weight and biochemical estimations (plasma glucose (PGL), triglycerides (PTG), total cholesterol (PTC), and LDL-c & HDL-c) were carried out just before and 7 days after the vehicle or STZ injection, i.e., on 3 weeks of dietary manipulation in rats. The rats with the fasting PGL of ≥200 mg/dl were considered diabetic and selected for further pharmacological wound healing studies. The feed and water intake of the animals were also measured.

The rats were allowed to continue to feed on their respective diets until the end of the study. The treatments of drugs were started after 7 days STZ injection on the confirmation of hyperglycemia in diabetic hyperlipidemic rats, it was considered as day 0 for further pharmacological activity.

Experimental design for Wound Healing Study:

Dead Space Wound (Granuloma Study) [24]:

Group-I: Normal control group receive oral 5ml/kg of 0.5% CMC with 0.1% Tween 80(Vehicle)

Group-II: DH control group receive oral 5ml/kg of 0.5% CMC with 0.1% Tween 80 (Vehicle)

Group-III: DH test group receive oral 5ml/kg of 200mg/kg *Murraya koenigii* extract.

Group-IV: DH test group receive oral 5ml/kg of 300mg/kg *Murraya koenigii* extract.

Group-V: DH test group receive oral 5ml/kg of 400mg/kg *Murraya koenigii* extract.

Group-VI: DH test group receive oral 5ml/kg of 10mg/kg Ciprofloxacin.

Wistar rats were divided into six groups as above, each containing six animals and anesthetized with 1 ml of intravenous ketamine hydrochloride (10 mg/kg body weight). Dead space wounds were inflicted by implanting sterile cotton pellets (10mg each), one on either side in the groin and axilla on the ventral surface of each rat by the technique of D'Arcy *et al*. as described by Turner [25].

Animals were treated daily with drugs as per theirs grouping, from 0 day to 9th post-wounding day. On the 10th post-wounding day, the granulation tissue formed on the implanted cotton pellets was carefully removed under anaesthesia. After noting the wet weight of the granulation tissue, the tissue was dried at 60^{o}C for 12 hr, and the dry granulation tissue weight was recorded. To the dried tissue 5 ml 6N HCl was added and kept at 110^{o}C for 24 hr. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline [26].

The assay procedure for Hydroxyproline Content [26]: Aliquots of standard hydroxyproline (2-20µg) prepared from stock solution and test samples containing hydroxyproline less than 10 µg/mL will be mixed gently with sodium hydroxide (2N final concentration) in a total volume of 50 µL. The sample will be hydrolyzed by autoclaving at 120°C for 20 min. 450 µL of chloramines-T will be added to the hydrolyzate, mixed gently, and the oxidation will be allowed to proceed for 25 min at room temperature. 500 µL of Ehrlich's aldehyde reagent will be added to each sample, mixed gently and the chromophore will be developed by incubating the samples at 65°C for 20 min. Absorbance of each sample will be re-added at 550nm using a spectrophotometer to find out concentration of hydroxyproline.

Statistical Analysis: Results were expressed as Mean ± Standard Deviation (SD). The data was statistically analyzed using the one-way ANOVA followed by Tukey-Kramer multiple comparison test to determine whether results in a particular group were significantly different from those in the corresponding control groups. Results were statistically significant when P values are less than 0.05 ($P < 0.05$).

RESULTS: The freshly prepared aqueous extract was subjected to preliminary phytochemical screening test for various constituents. This revealed the presence of alkaloids, tannins, saponins, flavonoids, terpenoids and steroids.

In acute toxicity studies, the extract in doses up to 2000mg did not produce any signs of toxicity and mortality. The animals were physically active and were consuming food and water in a regular way. No abnormal behavior was noticed. As no mortality was recorded within 24 hours during the acute toxicity test, LD_{50} could not be calculated.

In dead space wound model, animals treated with *Murraya koenigii* (oral administration of variable dosage level 200mg/kg, 300mg/kg and 400mg/kg) leaves aqueous extract showed significant ($P<0.01$) increase in Wet & Dry granulations tissue weight (biophysical parameter) and hydroxyproline content (biochemical parameter) when compared to the diabetic hyperlipidemic control group rats and standard Ciprofloxacin (10mg/kg) treated group showed significant ($P<0.05$) increase in Wet granulations tissue weight but not dry granulation tissue weight and hydroxyprolin content when compared to the diabetic hyperlipidemic control group rats.

In this study, very significant ($p<0.001$) result was found with 300mg/kg dose level because the effect was dose dependent up to 300mg equivalent of extract and there was no significant difference in between MK-300 & MK-400 mg/kg treated groups for Wet & Dry granulations tissue weight and hydroxyproline content (**Table 1, Figure 1 & 2**).

TABLE 1: EFFECT OF *MURRAYA KOENIGII* ON WET AND DRY GRANULATION TISSUE WEIGHT AND HYDROXYPROLIN CONTENT IN DEAD SPACE WOUND MODEL

Animal Group	Treatment	Wet tissue weight (mg/100gm rat)	Dry tissue weight (mg/100gm rat)	Hydroxyprolin content (mg/gm tissue weight)
Group-I	Normal control	106.90±5.45***	38.76±2.01***	34.87±2.14***
Group-II	DH control	84.77±4.63	32.13±1.83	27.65±1.58
Group-III	MK-200mg/kg	100.58±6.42**	37.35±2.02**	37.17±2.23***
Group-IV	MK-300mg/kg	166.32±7.89***	46.43±2.21***	63.38±3.51***
Group-V	MK-400mg/kg	175.22±8.02***,b	48.60±2.72***,b	67.65±3.57***,b
Group-VI	Ciprofloxacin 10mg/kg	97.03±5.07*	35.78±2.04 a	30.62±1.83 a

Values are expressed as Mean ± SD; n=6, ***P<0.001, **P<0.01 & *P<0.05 & 'a'- no significant' when compared with Diabetic hyperlidemic (DH) control group; 'b'- no significant when compare to MK-300 (group-IV), using one way ANOVA followed by Tukey-Kramer multiple comparison test.

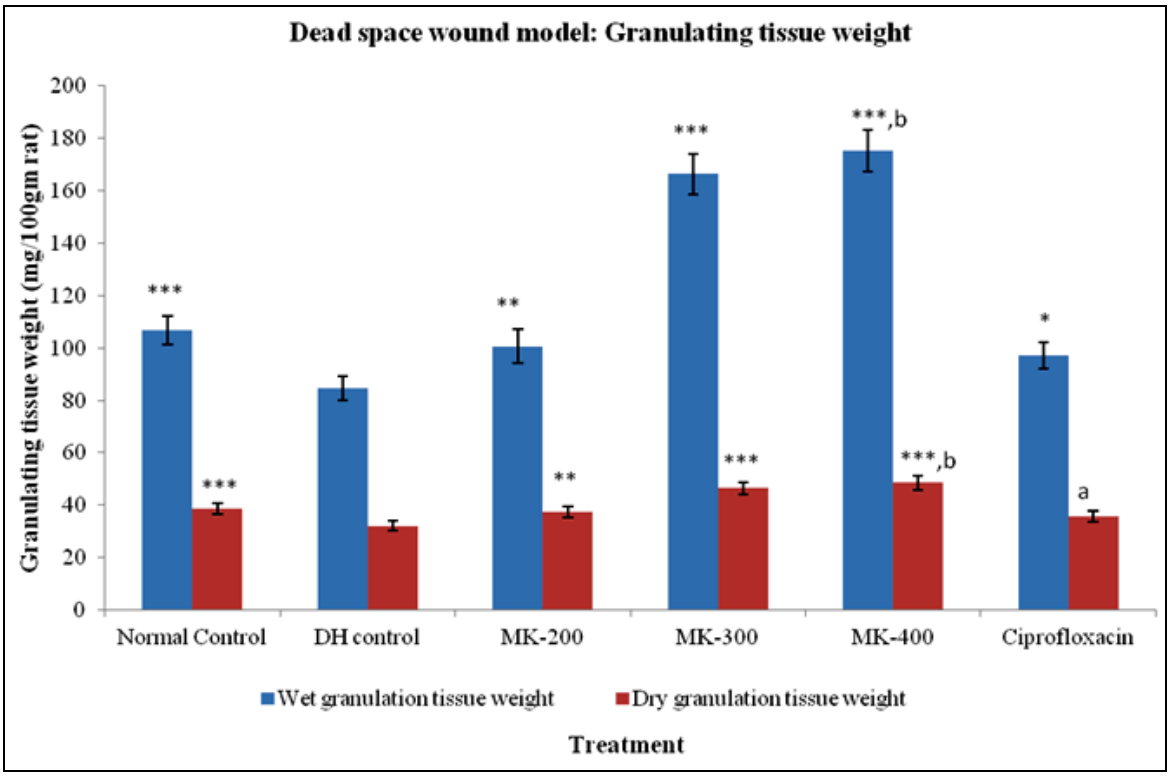

FIGURE 1: EFFECT OF *MURRAYA KOENIGII* ON WET AND DRY GRANULATION TISSUE WEIGHT IN DEAD SPACE WOUND MODEL

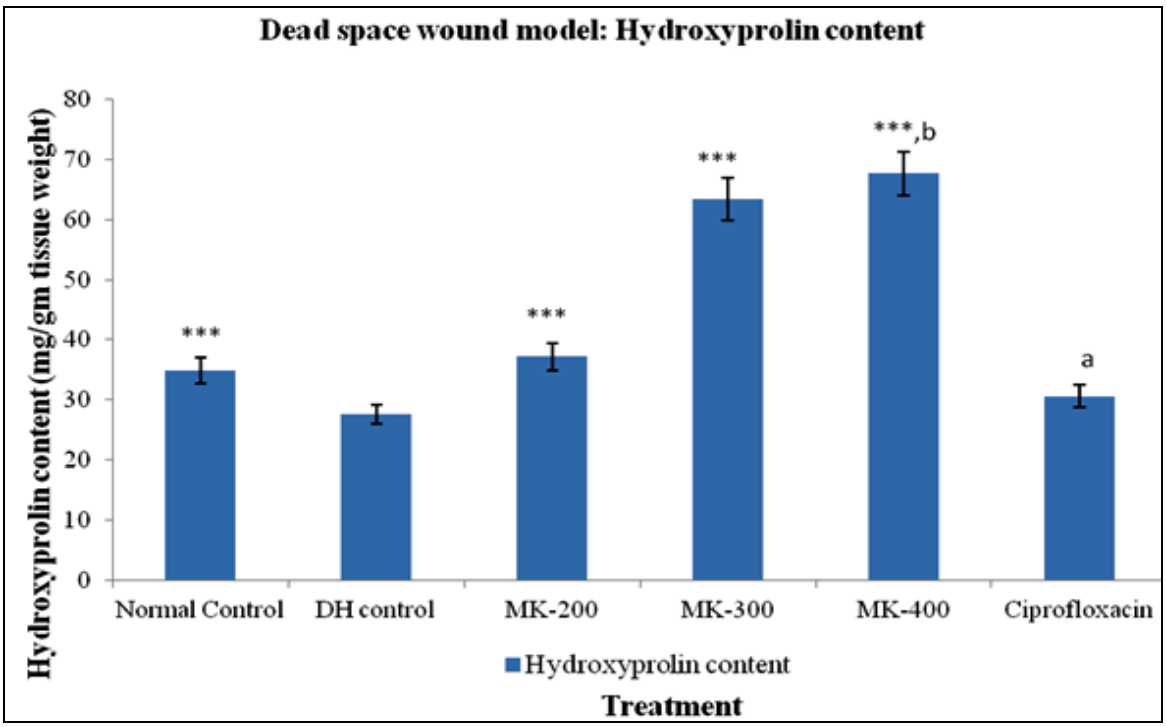

FIGURE 2: EFFECT OF *MURRAYA KOENIGII* ON HYDROXYPROLINE CONTENT IN DEAD SPACE WOUND MODEL

DISCUSSION & CONCLUSION: The present study was undertaken to evaluate whether *Murraya koenigii* leaves aqueous extract promote wound healing in experimentally induced wounds in rats. The results of the present study substantiate the use of *Murraya koenigii* leaves aqueous extract in folklore medicine for the treatment of wounds.

The oral administration of aqueous extract promoted the Wet & Dry granulation tissue weight and hydroxyproline content in dead space wound in diabetic hyperlipidemic albino Wistar rats. The acute toxicity study was done by "fixed dose" method according "OECD guidelines 420.

The extract in doses up to 2000mg did not produce any signs of toxicity and mortality. As no mortality was recorded within 24 hours during the acute toxicity test, LD50 could not be calculated. The non – toxic effect of the aqueous leaf extract of *Murraya koenigii* lend support to the widespread use of the plant as a spice for food flavoring. Collagenation, wound contraction and epithelization are crucial phases of wound healing. The phases of inflammation, macrophasia, fibroplasias and collagenation are intimately interlinked.

Thus, an intervention into any one of these phases by drugs could eventually either promote or depress one, other or all phases of healing. Growth hormone is known to promote the healing process by enhancing epithelial cell proliferation and cell collagen formation. Collagen is the family of protein, which provide structural support and it is the main component of tissue such as fibrous tissue and cartilage. The collagen synthesis is stimulated by various growth factors [27]. Growth hormone is also known to promote the proliferation of fibroblasts [28] and fibroblast proliferation form the granulation tissue.

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound and there is a rapid increase in the synthesis of this protein in the wound area soon after an injury, which provides strength and integrity to tissue matrix. Measurement of this hydroxyproline, which comes from the breakdown of collagen, has been used as an index of collagen turnover. In the present study, the aqueous extract of *M. koenigii* showed dose-dependent faster healing and increased the wet and dry granulation tissue weight (epithelial cells proliferation) and the hydroxyproline (collagen formation) content of the granulation tissue indicating the presence of higher epithelial cell proliferation and collagen content and its turnover leading to rapid healing wounds.

In this study, animals treated with *Murraya koenigii* leaves aqueous extract in variable dosage level showed significant ($P<0.01$) increase in Wet & Dry granulations tissue weight (biophysical parameter) and hydroxyl-proline content (biochemical parameter) when compared to the diabetic hyperlipidemic control group rats and standard Ciprofloxacin (10mg/kg) treated group showed significant ($P<0.05$) increase in Wet granulations tissue weight but not dry granulation tissue weight and hydroxyproline content when compared to the diabetic hyperlipidemic control group rats. The very significant ($p<0.001$) result was found with 300mg/kg dose level because the effect was dose dependent up to 300mg equivalent of extract.

We have demonstrated wound healing properties of *Murraya koenigii* application in streptozotocin induced diabetic and high cholesterol diet induced hyperlipidemia in rats.

Low dose of streptozotocin injection caused diabetes mellitus type-II, probably due to partial destruction of the cells of the Islets of Langerhans of the pancreas [29]. This results in over-production of glucose and decreased utilization by the tissues, forming the basis of hyperglycemia in diabetes mellitus [30]. The delay in diabetic wound healing, is due to interruption of cytokine release from macrophages, which may be due to the fundamental diabetic-hyperlipidemic condition

The preliminary phytochemical screening of extract of *Murraya koenigii* shows presence of mucilage, proteins, sterols and Triterpenoids, alkaloids, tannins, flavonoids, phenolic compounds. This wound healing activity of the extract observed might be due to the presence of phytochemicals present in the plant extract.

In conclusion, these preliminary investigations and data obtained from this study demonstrated that *Murraya koenigii* treatment increased granuloma tissue weight and hydroxyproline content in dead space wound in diabetic hyperlipidemic rats. The exact mechanism(s) by *M. koenigii* increased the granuloma tissue weight of granulation tissue and hydroxyprolin content in tissue cannot be explained with the present data.

ACKNOWLEDGEMENT: I take this privilege and pleasure to acknowledge the contributions of many individuals including my guide Prof (Dr.) Angshu Bandyopadhyay, Director, Sri Balaji College of Pharmacy, Jaipur, Management of CMJ University-Shillong and my wife Mrs. Priyanka Yadav, who have been inspirational and supportive throughout my work undertaken and endowed with the most precious knowledge to see success in my endeavor.

REFERENCES:

1. Evans P, The healing process at the cellular level. *Physiotherapy*.1983; *20*: 256.
2. Gupta N, Gupta S K, Shukla V K & Singh S P, An Indian community based epidemiological study of wounds, *J Wound Care*.2004;13:323.
3. Suguna L, Chandrakasan G & Joseph K T, Influence of honey on biochemical and biophysical parameters of wounds in rats. *J Clinic Biochem Nutr*, 1999; *14:91*.
4. Sharma S P, Aithal K S, Srinivasan K K, Udupa A L, Kumar V & Kulkarni D R, Anti-inflammatory and wound healing activities of the crude alcoholic extracts and flavanoids of *Vitex leucoxylon*. *Fitoterapia*, 1990; 61:263.
5. Chithra P, Suguna L & Chandrakasan G, Influence of arginine wound healing in rats, *J Clinic Biochem and Nutr*, 1995; *18:111*.
6. Falanga V, The Chronic wound: Impaired wound healing and solutions in the context of wound bed preparation, *Blood Cells Mol Dis, 2004;* 32:88.
7. Singer A J & Clark R A, Cutaneous wound healing, *N Engl J Med,* 1999; 341: 738.
8. Goodson W H & Hunt T K, Studies of wound healing in experimental diabetes Mellitus, *J Surg Res.1977;*22:221.
9. Doxey DL, Nares S, Park B, Trieu C, Cutler CW Lacopino AM. Diabetes-induced impairment of macrophage cytokine release in a rat model: potential role of serum lipids. Life sciences 1998; 673 (13):1127-36.
10. Kong YC, Ng KH, But PP, Li Q, Yu SX, Zhang HT, Cheng KF, Soejarto DD, Kan WS, Waterman PG. Sources of the antiimplantation alkaloid yuehchukene in the genus Murraya. Journal of Ethanopharmacology 1986; 15: 195-200.
11. Tee ES, Lim CL. Carotenoid composition and content of Malaysian vegetables and fruits by the AOAC and HPLC methods. Food Chemistry 1991; 41: 309- 339.
12. Chevalier. The encyclopedia of medicinal plants. London: Dorling Kindersley Publisher, London. 1996.
13. Sivarajan VV, Balachandran I. Ayurvedic drugs and their plant sources. Oxford and IBH Publishing Co. Ltd. New Delhi, 1994, pp. 199.
14. Khan BA, Abraham A, Leelamma S. Antioxidant effects of curry leaf, Murraya koenigii and mustard seeds, *Brassica juncea* in rats fed with high fat diet. Indian J. Exp. Biol. 1997; 35(2): 148-150.
15. Nakatani N. Phenolic antioxidants from herbs and spices. Biofactors. 2000; 13:141-146.
16. Kesari N, Gupta RK, Watal G. Hypoglycemic effects of *Murraya koenigii* on normal and alloxan-diabetic rabbits. Journal of Ethnopharmacology 2005; 97: 247- 251.
17. Arulsevan P, Subramanian SP. Beneficial effects of *Murraya koenigii* leaves on antioxidant defense system and ultrastructural changes of pancreatic beta cells in experimental diabetes in rats. Chem Biol Interact. 2006; 165: 155-164.
18. Kokate CK, Purohit AP, Gokhale. Practical Pharmacognosy. 2nd Edition, Nirali Prakashan, Pune. 1994: 54-60.
19. Evans WC, Trease. Text Book of Pharmacognosy. English language Book Society, 13th Edition, England. 289-388.
20. Raphel and Ikhan. Natural Products- A Lab guide. 12th Edition. 233-236. 34. Gosh MN. Fundamentals of Experimental Pharmacology. 3rd edn. Calcutta. Scientific Book Agency; Calcutta. 2005: 190-197.
21. OECD (2001) Guideline, 420, Acute Oral Toxicity – Fixed Dose Procedure: Environmental Health and Safety Monograph series on Testing and Assessment.
22. Guido S, Joseph T. Effect of chemically different calcium antagonists on lipid profile in rats fed on a high fat diet. Indian J Exp Biol 1992; 30 (4):292-4.
23. K. Srinivasan, B. Viswanad, Lydia Asrat, C.L. Kaul, P. Ramarao,. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. Pharmacological Research.2005; 52: 313–320.
24. B Shivananda Nayak, Lexley Pinto Pereira & Dale Maharaj., Wound healing activity of *Carica papaya* L. in experimentally induced diabetic rats. Indian Journal of Experimental Biology .2007; 45:739-743.
25. Turner R A, Inflammatory agent in screening methods of pharmacology. 2nd ed. (Academic Press, New York) 1965.
26. Neuman R E & Logan M A, The determination of hydroxyproline, J Biol Chem, 1950; 184: 299.
27. Corton S R, Kumar V & Collins T, Robbins pathologic basis of diseases, 6th edition, (Harcourt Limited, New Delhi, India), 2003, 96.
28. Williams T C & Frohman L A, Potential therapeutic indication for growth hormone releasing hormone in the condition other than growth retardation, *Pharmacotherapy*, 1986; 6: 311.
29. G. Kavalali, H. Tuncel, S. Goksel, M.H. Hatemi., Hypoglycemic activity of *Urtica pilulifera* in streptozotocin-diabetic rats. J Ethnopharmacol. 2002; 84, 241.
30. R. Chattopadhyay., Indian J Exp Biol., 1993; 3.

****