Regulation of wound strength by *Ocimum sanctum*: *in silico* and *in vivo* evidences

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INTRODUCTION

Wound healing is the process of repair that involves various cells and events follows injury to the skin and other soft tissues. The phases of normal wound healing include hemostasis, inflammation, proliferation and remodelling. Wound healing is directly related to interactions between cells and the components of the extracellular matrix (ECM). The ECM regulates the growth, proliferation, movement, and differentiation of the cells living within it. The ECM is composed of three groups of macromolecules: fibrous structural proteins, such as collagens and elastins that provide tensile strength and recoil; adhesive glycoproteins that connect the matrix elements to one another and to cells; and proteoglycans and hyaluronan that provide resilience and lubrication. Degradation of collagen and other ECM proteins is achieved by matrix metalloproteases, which consist of 23 distinct proteases in humans.¹ MMPs belong to four classes: the collagenases (MMP-1, -8 and -13), the gelatinases (MMP-2 and -9), thestromelysins (MMP-3, -10 and -11) and a heterogeneous group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26) and epilysin (MMP-28).² The collagenases are rapidly inhibited by a family of specific tissue inhibitors of metalloproteinases (TIMP) thus preventing uncontrolled action of these proteases. These TIMPs are a group of

ABSTRACT

Background: The present work has been an attempt to facilitate the scientific understanding of the wound strength by *Ocimum sanctum* (OS, holy basil) a traditional knowledge practiced since ancient times in India.

Methods: The *in vivo* Incision (wound strength) and Dead space wound models (biochemical estimation of components of ECM) in rats and *In silico* method, where one of the target proteins from each class of MMPs involved in wound strength was selected for molecular docking with eugenol (one of the flavonoid present in OS).

Results: Molecular docking showed that eugenol was able to inhibit all selected MMPs, i.e. collagenase (-6.37 Kcal/mol), gelatinase (-5.99 Kcal/mol), elastase (-6.31 Kcal/mol) and stromelysin (-5.79 Kcal/mol). Ethanolic extract of *Ocimum sanctum* (OSE, 200-800 mg/kg) when administered as suspension showed dose-dependent increase in wound breaking strength in *in vivo* Incision wound rat model. OSE 400 mg/kg produced a significant increase in protein and collagen constituents like hydroxyproline, hexuronic acid and hexosamine in the connective tissue content of extracellular matrix when studied in Dead space wound model in rat.

Conclusions: The present study is an attempt to correlate the *in vivo* findings on wound strength promoting activity by *Ocimum sanctum* with *in silico* tools.

Keywords: Eugenol, MMP, Collagenase, Gelatinase, Elastase, Stromelysin, Wound healing
four proteins that as a group effectively inhibit all MMPs in vivo. TIMP have cell growth promoting activity for many types of cells and protect cells from apoptosis.\(^4\)

Wound strength is a balance between collagen synthesis and degradation. Recovery of tensile strength depends not only on increased collagen synthesis but also on decreased degradation. In acute wounds, there is a balance between protease activity and ECM deposition.\(^4\) However, excessive MMP activity contributes to the development of chronic wounds. Delayed healing is characterized by an increase in matrix metalloproteinases (MMPs), a decrease in the TIMPs.\(^5\) Recent evidence indicates that the anticoagulant, activated protein C may be useful in the treatment of non-healing wounds by preventing excessive protease activity through inhibition of inflammation.

The role of collagen breakdown in wound healing is still not well understood. Selective control of MMP activity may prove to be a valuable therapeutic approach to promote healing of chronic ulcers. The majority of the clinical trials using synthetic metalloproteinase inhibitors (Batimastat) were conducted and proved unsuccessful due to side effects. Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants is because they constitute a rich source of bioactive chemicals that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, glycosides, flavonoids and essential oils. Today the large numbers of drugs in use are derived from plants, like artemether, paclitaxel, podophyllotoxins, vinblastine, vincristine and many others. The leaves from Ocimum sanctum has long been considered to have natural medicinal properties, for instance, allergic disorders, anticarcinogenic, anthelmintic, antiseptic, antirheumatic, and antibacterial properties and immunomodulatory properties.\(^6\)

Bioinformatics represents a new growing area of science that uses computational approaches to understand biomedical problems. The aim of the present study was to determine the role of Ocimum sanctum in wound healing by in vivo and in silico methods. The in vivo evaluation of wound strength and biochemical estimation of components of ECM was done using Incision and Dead space wound models in rats. Both are reproducible and simple models sharing many characteristics with human wound. In in silico evaluation, the one target protein from each class of MMP, which involved in wound strength, was selected. Molecular docking was done to evaluate the molecular interaction of eugenol, to these MMPs.

**METHODS**

**Collection and preparation of plant extract**

100 g of OS leaves powder was extracted with 500 ml of ethanol and was kept for 3 days at room temperature and the extract was filtered. The above procedure was repeated twice and the 50% ethanolic extracts (OSE) so obtained were mixed and dried at room temperature. The yield was about 8.7% (w/w). OSE was stored at -20°C until further use.

**In vivo method**

**Animals**

Inbred Charles-Foster (CF) albino rats (150-250 g) and mice (25-30 g) of either sex was obtained from the central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. They were kept in the departmental animal house at 26 ± 2°C and relative humidity 44-56%, light and dark cycles of 10 and 14 h respectively for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet and water ad libitum. ‘Principles of laboratory animal care’ (NIH publication no. 82-23, revised 1985) guidelines were followed. Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work (Notification No. – Dean/2008-09/316 dated 5/1/2009).

**Treatment protocol**

The wound healing study was undertaken in incision wound and dead space wound models. OSE and the standard drug Vitamin E (VTE) were suspended in 1% carboxy methyl cellulose (CMC) in distilled water and given orally once daily from day 1, 4 hour after the induction of experimental wounds for 10 days while, control rats received 1% CMC only. The animals received OSE/ VTE, orally with the help of an oro gastric tube as water suspension in the volume of 10 ml/kg body weight. Graded doses of OSE (200, 400 and 800 mg/kg) were given to find the optimal effective dose of OSE enhancing the wound breaking strength in incision wound model in rats. VTE (Eviron, Merck Limited) promoting wound healing was given in the dose of 200mg/kg as standard drug for the comparison of wound healing actions in experimental animals.

**Incision Wound**

Two paravertebral incisions (6 cm long) were made through the full thickness of the skin on either side of the vertebral column. Wounds were closed with interrupted sutures, 1 cm apart. The sutures were removed on the 7th day. Wound breaking strength (WBS) was measured on the 10\(^{th}\) post-wounding day. WBS was measured in anesthetized rats secured on to the operation table. A line was drawn on either side of the paravertebral wound 3mm away from the wound. Two alliice forceps were firmly applied on to the line facing each other. One of the forceps was fixed, while the other was connected to a freely suspended lightweight polypropylene graduated container through a string run over to a pulley. Water was allowed to flow from the reservoir slowly and steadily into the container. A gradual increase in weight was
transmitted to the wound site pulling apart the wound edges. As and when the wound just opened up, the water flow was arrested and the volume of water collected in the container was noted. Three readings were recorded for a given incision wound and the procedure was repeated on the wound on the contra lateral side. The average reading of the group was taken as an individual value of breaking strength.  

**Dead Space Wound**

These wounds were created by implanting two polypropylene tubes (0.5 x 2.5 cm² each), one on either side in the lumbar region on the dorsal surface of each rat. On the 10th post-wounding day, the animals were sacrificed and granulation tissue formed on the implanted tubes was carefully dissected out for the estimation collagen content in the granulation tissue.  

**Estimation of protein and collagenses**

The granulation tissues from one of the tubes were collected and weighed. 5 mg of tissue was used for the estimation of protein while the rest of tissue was taken in glass stopper test tubes. 6N HCl was added in each tube so that it contained 40 mg of the granulation tissue per ml of HCl. The tubes were kept on boiling water bath for 24 hours (12 hours each for two days) for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralised by 10N NaOH using phenolphthalein. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline, hexuronic acid and hexosamine following the standard procedures.  

**In silico**

The structure of the MMPs and eugenol were retrieved from PDB and PubChem respectively. Molecular docking was done by AutoDock4. Autodock actually consists of two main programs Autodock and Auto Grid. Autodock performs the docking of the ligand to a set of grids describing the target protein. Lamarckian Genetic Algorithm is comprised of a stochastic population generator, a docking routine based on a Lamarckian genetic algorithm, and a local search function based on molecular mechanics (MM) energy minimization. The input files for Auto Grid and Autodock were created, and then the grid map calculation was run, followed by docking calculation in Autodock. These grid maps were used for Autodock docking calculations to determine the total interaction energy for a ligand with a macromolecule. The grid box size was set at 126, 126 and 126 A° (x, y, and z) to include all the amino acid residues that present in rigid macromolecules. The spacing between grid points was 0.375 angstroms. The Lamarckian Genetic Algorithm (LGA) was chosen search for the best conformers. During the docking process, a maximum of 10 conformers were considered. The population size was set to 150 and the individuals were initialized randomly. Maximum number of energy evaluation was set to 500000, maximum number of generations 1000, maximum number of top individual that automatically survived set to 1, mutation rate of 0.02, crossover rate of 0.8, Step sizes were 0.2 Å for translations, 5.0° for quaternions and 5.0° for torsions. Cluster tolerance 0.5Å, external grid energy 1000.0, max initial energy 0.0, max number of retries 10000 and 10 LGA runs were performed. Autodock results were analyzed to study the interactions and the binding energy of the docked structure. Visualization of docked structure was done using PYMOL tool.  

**Statistical analysis**

Statistical comparison was performed by SPSS using one way analysis of variance (ANOVA).  

**RESULTS**

**In vivo study**

**Incision wound model- study of Wound breaking strength (WBS)**

Control rats receiving 1% CMC orally showed WBS as 338.3 ± 13.5 g. OSE 200, 400 and 800 mg/kg showed WBS as 400.8 ± 7.6, 458.3 ± 7.0g and 453.3 ± 8.7g respectively while VTE 200 mg/kg showed WBS as 528.3 ± 6.9 g. All the doses of OSE and VTE showed significant increase in WBS as compared with control; therefore OSE 400 mg/kg was selected for further study (Table 1).  

**Dead space wound model- study of collagen determinants**

Both OSE and VTE significantly enhanced dried weight of tissue per 100 g body weight of rats as well as protein content mg/g of tissue (Table 1). Similarly collagen determinants like hydroxylproline, hexuronic and hexosamine were also significantly increased after treatment with OSE which was comparable with VTE (Table 1).  

**In silico study**

The target protein structure of collagenase, gelatinase, elastase and stromelysin were docked with eugenol, which provided excellent Auto Dock results as were seen by their least values of the binding energy (Table 2). The best possible binding modes of the eugenol at four targeted protein’s active sites are displayed in figures (1-4) and their corresponding energy values and inhibitory constants are listed in Table 2.
Table 1: Effect of 50% ethanolic extract of dried leaves of *Ocimum sanctum* (OSE) and Vitamin E (VTE) on wound breaking strength (WBS, Incision model) and granulation tissue weight, protein, hydroxyproline (HXPR), hexuronic acid (HXUA) and hexosamine (HXAM) per mg protein contents (Dead space wound model) in rats.

| Oral treatment (mg/kg, od x 10 days) | Incision model | Dead space wound model |
|-------------------------------------|----------------|------------------------|
|                                     |                | WBS (g)                | Dried weight (g/100 g bw) | Protein (mg/g dried tissue) | Collagen determinants (μg/mg protein) | Granulation tissue parameters |
| Control (1% CMC)                    |                | 338.3 ± 13.5           | 226.7 ± 9.89               | 81.5 ± 2.52                 | 142.8 ± 6.56                          | 87.9 ± 8.68             |
| OS (400)                            |                | 458.3 ± 7.0            | 301.7 ± 10.1              | 95.3 ± 2.22                 | 196.8 ± 12.2                          | 122.8 ± 6.14           |
| VTE (200)                           |                | 528.3 ± 6.9            | 310.0 ± 8.56              | 95.8 ± 2.47                 | 192.5 ± 17.8                          | 129.6 ± 8.89           |

Results are mean ± SEM of 6 rats in each group.

*Table 2: Auto Dock results of eugenol with collagenase, gelatinase, elastase and stromelysin.*

| Sr. No. | Target protein | Ligand | No. of H-bond | Docked residue | Binding energy (Kcal/mol) | Inhibition Constant (µM) |
|---------|----------------|--------|---------------|----------------|--------------------------|-------------------------|
| 1       | Collagenase (MMP-8) (1MNC) | Eugenol | 2             | Ala241/HN, Ala241/O | -6.37                   | 21.38                   |
| 2       | Gelatinase (MMP-2) (1QIB)  | Eugenol | 1             | Thr227/O, Ala241/O | -5.99                   | 40.91                   |
| 3       | Elastase (MMP-12) (1HNE)  | Eugenol | 1             | Arg128/O         | -6.31                   | 43.98                   |
| 4       | Stromelysin (MMP-3) (2DIO) | Eugenol | 1             | Ala165/O         | -5.79                   | 57.24                   |

Result of docking analysis of human collagenase showed the binding site as two hydrogen bond at Ala241/HN and Ala241/O for eugenol (Figure 1). Figure 2, showed the binding interaction of gelatinase with eugenol, wherein one hydrogen bond at Thr227/O was found to interact with eugenol. Docking analysis of the human elastase with eugenol showed binding interaction of elastase-eugenol, with one hydrogen bond at Arg128/O positions (Figure 3) while, stromelysin showed binding interaction of one hydrogen bond at Ala165/O with eugenol (Figure 4).

**Molecular interaction study**

![Figure 1: Molecular docking study of collagenase with eugenol.](image1)

![Figure 2: Docking study of gelatinase with eugenol.](image2)

![Figure 3: Molecular docking study of elastase with eugenol.](image3)
DISCUSSION

In vivo study showed that Ocimum sanctum extract increased tensile strength as compared to control. In wound breaking strength model, the increase in tensile strength of treated wounds may be due to the increase in collagen concentration (Increased expression or decreased breakdown of collagen) and stabilization of the fibres. Since incisional wounds treated with the Ocimum sanctum showed slightly greater tensile strength, it may be inferred that it not only increased collagen synthesis per se, but it could also because of decreased breakdown of collagen. Cutaneous wound healing is a complex and dynamic process, which involves the coordinated and sequential deposition of extracellular matrix molecules, leading to the formation of a resistant new tissue.

Among these molecules, glycosaminoglycans (GAG) and proteoglycans (PG) are, with collagens and fibronectin, the major components of the connective tissue extracellular matrix. In addition to their structural functions, GAG and PG play a part in several processes in relation to wound healing, such as cellular adhesion, migration, and proliferation. In dead space wound model there was increase in protein content of granulation tissue this could be either due to decreased proteolytic activity because of protease inhibition or by increased expression of matrix proteins. Assessment of collagen content in granulation tissue of control and experimental wounds by estimating collagen determinants like hydroxyproline, hexuronic acid and hexosamine clearly revealed that OS enhanced the collagen synthesis and deposition and also found to inhibit degradation. In the wound healing process, it was found that collagen formation peaks at day 7 and epithelialisation occurs in 48 h under optimal conditions. Proteoglycans form large complexes, both to other proteoglycans, to hyaluronan and to fibrous matrix proteins. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline, therefore, has been used in the present study as an index of collagen turnover. The data depicted in table 1, in results section showed that the hydroxyproline content of the granulation tissue of the animals treated with OS was significantly increased when compared to the control group of animals and therefore indicates the increased collagen turnover. This increase was significantly higher than control in terms of absolute value as well as per mg protein value. The increased hydroxyproline content of the dead space wounds was found to indicate faster collagen turnover leading to rapid healing with concurrent increase in the tensile strength of the treated wounds. Hexosamine and hexuronic acid are matrix molecules, which act as ground substratum for the synthesis of new extracellular matrix. In the present study, hexuronic acid and hexosamine concentrations which are the component of glycosaminoglycans were found to be significantly increased in the extract when compared with control. The glycosaminoglycans are known to stabilize the collagen fibers by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characteristic size. Biochemical analysis of the wound granulation tissue confirmed that Ocimum sanctum extract increased GAG and protein deposition. As the Proliferative phase progresses, the TGF-ß decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor, tissue inhibitor of metallo-protease (TIMP).  

MMP-8 has a stronger affinity toward type I collagen and involved in various inflammatory processes. Docking studies revealed that eugenol may decrease collagenase activity by binding with MMP-8, which in turn increases the collagen 1 content of the extracellular matrix. This was evident by in vivo data of present study wherein increase in protein content was observed in case of ocimum sanctum treated group. The findings are in coherence with the results obtained on the MMP inhibition in wound healing study. There are reports on delayed wound healing in case of increased MMP activity.

In a recent clinical study, the wounds were examined for presence of MMPs and TIMPs. There was increased MMPs level and decreased TIMP level in the diabetic wound. Decrease in TIMPs in non-healing wound was reported in other studies as well.

MMP-2 has ability to proteolytically degrade gelatine, type I, IV and V collagens, elastin and vitronectin. Shapiro et al. (2010) have demonstrated that MMP-2 induces apoptosis in endothelial cells and inhibits neovascularization. In the present study, docking of eugenol with gelatinase showed binding interaction with three amino acid residues, Leu164/HN, Ile 222/O and Glu202/OE2 respectively. These binding may increase the level of gelatine, type I, IV and V collagens, elastin content of extracellular matrix. Similar type of finding in delayed wound healing with increased MMP activity were also made by various research groups.

A major substrate for MMP-12 is elastin, but MMP-12 is capable of degrading other ECM constituents. Elevated MMP-12 levels have been measured in various diseases. By inhibiting elastase, Eugenol may increase the amount of elastin which is important for wound strength.
Excessive and prolonged expression and activation of MMPs are etiologic causes of chronic diseases due, at least in part, to excessive tissue destruction. The result made in present study showed that eugenol can bind with MMP-12 and may decrease the activity of MMP-12.

The substrate specificity of MMP-3 is broad and MMP-3 has been described to degrade many ECM proteins such as fibronection, denatured collagens, laminin and proteoglycans. MMP-3 is incapable of degrading triple-helical collagens, but can cleave the globular portion of type IV collagen. MMP-3 seems to have a pro-apoptotic effect. MMP-3 has been described as an important factor in impaired wound healing. Both collagenase-1 and stromelysin-1 are found in fibroblasts underlyning the non-healing epithelium, and stromelysin-1 expression is especially prominent. There was increased stromelysin-1 and -2 and TIMP in acute and chronic wound but there was decreased TIMP in chronic wound suggesting the decreased levels of MMP inhibitor in chronic wound. Eugenol by binding with stromelysin may act as an exogenous substitute of TIMP.

CONCLUSION

Enzymes are major drug targets in drug discovery and development processes in the pharmaceutical and biotechnology industry. In wound healing process MMPs plays an important role. Over-expression and activation of MMPs has been linked to a range of diseases which include osteoarthritis, tumor metastasis, angiogenesis, cardiovascular diseases and chronic wounds. The present study emphasizes on the development of MMP inhibitors as therapeutic agents in wounds. Ocimum sanctum extract found to increase wound strength and this increase in wound strength may be because of inhibition of MMPs by eugenol, a constituent present in Ocimum sanctum. This increase in wound strength might also be because of increased level of different extracellular proteins like collagen and glycosaminoglycans. Thus, the present study systematically connecting the traditional knowledge available on Ocimum sanctum (Holy basil) for wound healing to the findings made by in silico and in vivo experiments. This effort may help in building the possibilities of developing novel drug to treat diverse diseases caused by excessive MMPs activities.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: Approval was taken from the institutional animal ethics committee

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doi:10.5455/2319-2003.ijbcp20140223

Cite this article as: Singh A, Mishra A, Verma S, Purohit V, Goel RK. Regulation of wound strength by Ocimum sanctum: in silico and in vivo evidences. Int J Basic Clin Pharmacol 2014;3:179-85.