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MMPs as Molecular Targets for Wound Healing by *Musa sapientum*: *In-silico* and *In-vivo* Evidences.

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ABSTRACT

The present work has been an attempt to study the wound healing by *Musa sapientum* (MS, banana). 50% ethanolic extract of *Musa sapientum* (MSE, 50-200 mg/kg) when administered as suspension showed dose-dependent increase in wound breaking strength in *in vivo* Incision wound rat model. MSE 100 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. The role of leucocyanidin, an active constituent of MS was evaluated, in wound healing by *in silico* methods. In *in-silico* study leucocyanidin was taken for evaluation as MMPs inhibitor by molecular docking. Molecular docking showed that leucocyanidin was able to inhibit all selected MMPs, i.e. collagenase (-9.67 Kcal/mol), gelatinase (-8.67 Kcal/mol), elastase (-8.27 Kcal/mol) and stromelysin (-10.17 Kcal/mol).

INTRODUCTION

The aim of wound healing is to correct structure and function of an injured tissue. The interactions between cells and the components of the extracellular matrix (ECM) are responsible for tissue repair. The ECM regulates the growth, proliferation, movement, and differentiation of the cells living within it. The ECM is composed of fibrous structural proteins (collagens and elastins), adhesive glycoproteins and proteoglycans. Degradation of structural proteins is achieved by matrix metalloproteases. MMPs belong to four classes: the collagenases (MMP-1, 8 and -13), the gelatinases (MMP-2 and -9), the stromelysins (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) [1]. The MMPs 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 four proteins that as a group effectively inhibit all MMPs in vivo[2]. TIMP have cell growth promoting activity for many types of cells and protect cells from apoptosis[3].

Recovery of tensile strength depends not only on increased collagen synthesis but also on decreased degradation. Wound strength is a balance between collagen synthesis and 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 [5]. Delayed healing is characterized by an increase in matrix metalloproteinases (MMPs), a decrease in the TIMPs [6] . Recent evidence indicates that the anticoagulant, activated protein C may be useful in the treatment of non-healing wounds by preventing excessive protease activity [5].

Selective control of MMP activity may 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 untoward side effects[7]. Herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe. The most important of these bioactive constituents of plants are alkaloids, tannins, glycosides, and flavonoids. The fruit pulp from unripe *Musa sapientum* has considered to have natural medicinal properties, for instance, peptic ulcer [8] , ulcerative

colitis and diabetes. A natural flavonoid from the unripe banana (*M. sapientum* var. *paradisiaca*) pulp, leucocyanidin, protects the gastric mucosa from erosions[9].

The aim of present study is to explore computational approach to understand the molecular mechanism of wound healing effects of 50% ethanolic extract of MS (MSE) using i) *in vivo* Incision (wound strength) and Dead space wound models (biochemical estimation of components of ECM) in rats and ii) *In silico* method, where one of the target proteins from each class of MMPs involved in wound strength was selected for molecular docking with leucocyanidin (one of the flavonoid present in MS). *In silico* toxicity, pharmacokinetic and drug likeliness score of leucocyanidin, was estimated to explore leucocyanidin as a lead compound.

MATERIALS AND METHODS

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 ± 2^0 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).

Collection and preparation of plant extract

The fruit pulp of *Musa sapientum* (MS) was collected during the months of September-March. 100 g of MS fruit pulp 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 extracts (MS) so obtained were mixed and dried at room temperature. The yield was about 3.0% (w/w). MSE was stored at -20°C until further use.

Treatment protocol

The wound healing study was undertaken in incision wound and dead space wound models. MSE 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 MSE/ VTE, orally with the help of an oro-gastric tube as suspension in the volume of 10 ml/kg body weight. Graded doses of MSE (50, 100 and 200 mg/kg) were given to find the optimal effective dose of MSE enhancing the wound breaking strength in incision wound model in rats. VTE (Evion, 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[10]

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 Allice 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[10]

These wounds were created by implanting two polypropylene tubes (0.5 x 2.5 cm^2 each), one on either side in the lumbar region on the dorsal surface of each rat. On the 10^{th} 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 collagens

The granulation tissues from one of the tubes were collected, weighed and dried at 40° C. 5 mg of dried material was used for the estimation of protein while the rest of dried material was taken in glass stopper test tubes. 6*N* HCl was added in each tube so that it contained 40 mg of the dried 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 10*N* 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[11], hexuronic acid[12] and hexosamine[13] following the standard procedures.

Acute toxicity study

10x of effective dose of MS (2000 mg/kg) suspended in 1% carboxymethylcellulose (CMC) in distilled water (1 ml/100 g body weight) were administered orally to the test group while control group received 1% CMC orally (6 mice of both sexes in equal number in each group). The acute toxicity studies were conducted as per the OECD guidelines 420 (OECD 2000) given by Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA). Observations were made at 2, 4 and 8 hrs after treatment for bodyweight, respiration rate, heart rate and behavioral signs like apathy, reduced locomotor behavior, any ANS (salivation, lacrimation, any colour change in eyes, mucous membrane, skin or fur), CNS (motor activity, convulsions, coma, behavioural pattern including level of consciousness and gait changes) activities and mortality after single dose test drug administration.

In Silico

The structure of the MMPs and leucocyanidin 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 0.5Aº, 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[14-17]. Visualization of docked structure was done using PYMOL tool[18].

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. MSE 50, 100 and 200 mg/kg showed WBS as 363.3 ± 6.8, 445.0 ± 9.7 and 438.3 ± 8.4g respectively while VTE 200 mg/kg showed WBS as 528.3 ± 6.9 g. All the doses of MSE and VTE showed significant increase in WBS as compared with control; therefore MSE 100 mg/kg was selected for further study (Table 1).

Dead space wound model- study of collagen determinants

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

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Table 1: Effect of 50% ethanolic extract of fruit pulp of *Musa sapientum* (CLE) 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				
		Granulation tissue parameters				
	WBS (g)	Dried weight (g/100 g bw)	Protein (mg/g dried tissue)	Collagen determinants (µg/mg protein)		
				HXPR	HXUA	HXAM
Control (1%CMC)	338.3 ± 13.5	226.7 ± 9.89	81.5 ± 2.52	142.8 ± 6.56	21.2 ± 2.47	87.9 ± 8.68
MSE (100)	445.0 ± 9.7[c]	290.0 ± 4.47 [b]	98.4 ± 4.34b	188.5 ± 18.1 a	52.5 ± 4.92 c	120.1 ± 5.90 b
VTE (200)	528.3 ± 6.9 [c]	310.0 ± 8.56 [c]	95.8 ± 2.47 b	192.5 ± 17.8 a	50.8 ± 4.47 c	129.6 ± 8.89b

Results are mean ± SEM of 6 rats in each group.
[a] P< 0.05, [b] P< 0.01 and [c] P< 0.001 compared to respective control group (Statistical analysis was done by one way analysis of variance by SPSS).

Acute toxicity study

The result of the acute toxicity study even with 10 times the effective dose of MSE did not show any change in ANS, CNS or mortality in mice till 14 days of study indicating it to be safe.

Auto Dock results of leucocyanidin with Collagenase, Gelatinase, Elastase and Stromelysin

The target protein structure of Collagenase, Gelatinase, Elastase and Stromelysin were docked with leucocyanidin, which provided excellent AutoDock results as were seen by their least values of the binding energy. The best possible binding modes of the leucocyanidin at four targeted protein's active sites are displayed in Fig. 1 a, b, c and d and their corresponding energy values and inhibitory constants are listed in Table 2. Result of docking analysis of human Collagenase showed the binding site as one hydrogen bond at Glu 219/OE2, Pro238/O and Ala 182/HN for leucocyanidin (Fig. 1a). Figure 1b showed the binding interaction of Gelatinase with leucocyanidin, wherein three hydrogen bond at Leu164/HN, Ile 222/O and Glu202/OE2 were found to interact with leucocyanidin. Docking analysis of the human Elastase with leucocyanidin showed binding interaction of Elastase- leucocyanidin, with three hydrogen bond at Ala232/HN, Arg128/O and His210/O positions (Fig. 1c) while, Stromelysin showed binding interaction of three hydrogen bond at Asn 162/O, Glu 202/OE2 and Leu218/O with leucocyanidin (Fig. 1d).

Table 2: Auto Dock results of Leucocyanidin with Collagenase, Gelatinase, Elastase and Stromelysin

S. No	Target protein	Ligand	No. of H bond	Docked residue	Binding energy Kcal/mol	Inhibition Constant (nM)
1	Collagenase (MMP-8) (1MNC)	Leucocyanidin	3	Glu219/OE2 Pro238/O Ala 182/HN	-9.67	81.54
2	Gelatinase (MMP-2) (1QIB)	Leucocyanidin	3	Leu164/HN Ile 222/O Glu202/OE2	-8.67	438.74
3	Elastase (MMP-12) (1HNE)	Leucocyanidin	3	Ala232/HN Arg128/O His210/O	-8.27	864.49
4	Stromelysin (MMP3) (2DIO)	Leucocyanidin	3	Asn 162/O Glu 202/OE2 Leu218/O	-10.17	35.07

DISCUSSION

In wound breaking strength model, the increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of the fibres. *In vivo* study showed that *Musa sapientum* extract increased tensile strength as compared to control. Since incisional wounds treated with the MS showed slightly greater tensile strength, it may be inferred that it could be due to increased collagen synthesis, decreased degradation and cross linking of the protein. 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[19]. Among these molecules, glycosaminoglycans (GAG) and proteoglycans (PG) are, with collagens and fibronectin, the major components of the connective tissue extracellular matrix[20]. In addition to their structural

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functions, GAG and PG play a part in several processes in relation to wound healing, such as cellular adhesion, migration, and proliferation[21].

Molecular interaction study

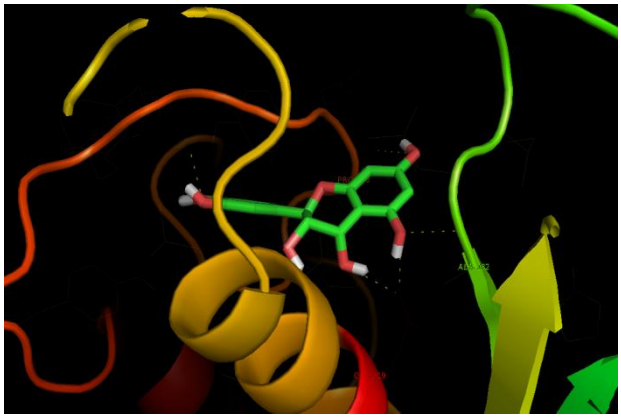

Fig. 1a. Molecular docking study of collagenase with leucocyanidin

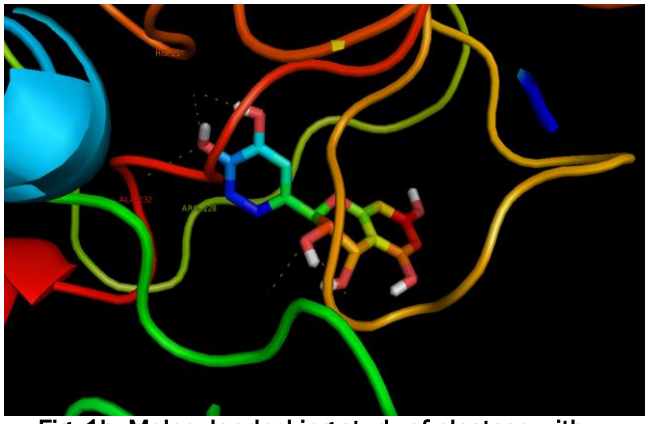

Fig. 1b. Molecular docking study of elastase with leucocyanidin

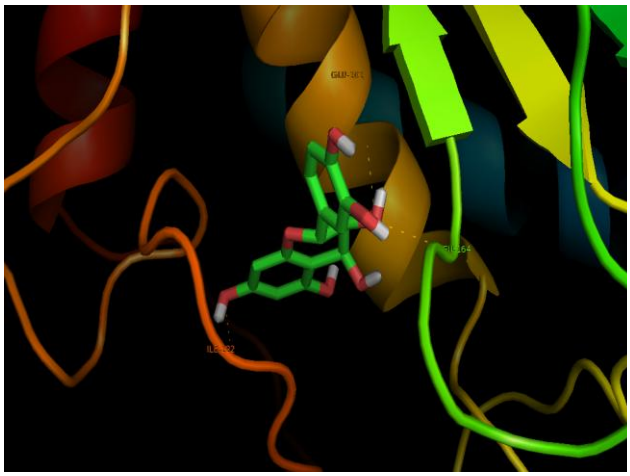

Fig.1c. Docking study of gelatinase with leucocyanidin

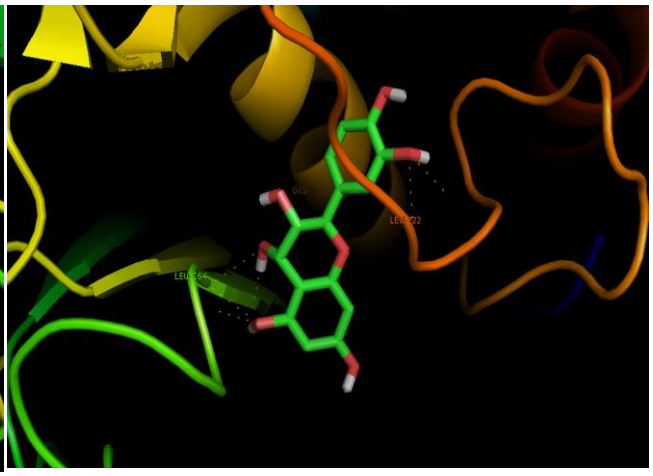

Fig. 1d. Docking study of stromelysin with leucocyanidin

Fig. 1a, showed the result of docking analysis of human Collagenase with leucocyanidin representing the binding site of the Collagenase as three hydrogen bond at Glu219/OE2, Pro238/O and Ala 182/HN. Fig. 1b, showed the binding interaction of Gelatinase-with leucocyanidin, wherein three hydrogen bond at Leu164/HN, Ile 222/O and Glu202/OE2 were found to interact with leucocyanidin. Fig. 1c., illustrated the docking analysis of the human Elastase with leucocyanidin showing binding interaction of Elastase- leucocyanidin, with three hydrogen bond at Ala232/HN, Arg128/O and His210/O. Stromelysin showed binding interaction of three hydrogen bond at Asn 162/O, Glu 202/OE2 and Leu218/O with leucocyanidin (Fig. 1d)

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 MS enhanced the collagen synthesis and deposition and also found to inhibit degradation. 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 MS 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 *Musa sapientum* 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)[22].

MMP-8 has a stronger affinity toward type I collagen and involved in various inflammatory processes[23]. Docking studies revealed that leucocyanidin 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 *Musa sapientum* 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[24,25].

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[6, 26].

MMP-2 has ability to proteolytically degrade gelatine, type I, IV and V collagens, elastin and vitronectin[27]. Shapiro et al. (2010)[28] have demonstrated that MMP-2 induces apoptosis in endothelial cells and inhibits neovascularization. In the present study, docking of leucocyanidin 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[29,30].

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[31]. By inhibiting elastase, leucocyanidin 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[32]. The result made in present study showed that leucocyanidin 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 fibronectin, denatured collagens, laminin and proteoglycans. MMP-3 is incapable of degrading triple-helical collagens, but can cleave the globular portion of type IV collagen[33]. MMP-3 seems to have a pro-apoptotic effect[34]. MMP-3 has been described as an important factor in impaired wound healing[35]. Both collagenase-1 and stromelysin-1 are found in fibroblasts underlying the nonhealing epithelium, and stromelysin-1 expression is especially prominent[36]. 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[37]. leucocyanidin 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. *Musa sapientum* extract found to increase wound strength and this increase in wound strength may be because of inhibition of MMPs by leucocyanidin, a constituent present *in Musa sapientum*. This increase in wound strength might also be because of increased level of different extracellular proteins like collagen and glycosaminoglycans. Thus, the present study is an attempt to correlate the *in vivo* findings on wound healing by *Musa sapientum* (banana) with *in silico* tools. These efforts may help in building the possibilities of developing novel drug to treat diverse diseases caused by excessive MMPs activities.

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