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Research Article

WOUND HEALING ACTIVITY FROM THE LEAF EXTRACTS OF *MORUS LAEVIGATA* AND *IN SILICO* BINDING STUDIES FROM ITS ISOLATES WITH GSK 3-β

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ABSTRACT

Background: Morus plant species possess enormous importance in the field of medicine and *Morus laevigata*, a white variety of genus Morus, is a popular hybrid species which is highly appreciated for its medicinal properties.
Methods: The present investigation is aimed to study the effect on wound healing capacity of different solvent extracts of *Morus laevigata*. Further, In-silico binding studies were carried out for a different drug candidates isolated from Morus laevigata taking nitrofurazone as a standard reference and CHIR-98014 as a positive control against glycogen synthase kinase 3-β (GSK3- β) protein.
Results: The results of the wound healing studies revealed that the methanolic extract has better wound healing activity in terms of mean time of epithelization (18.53±0.33 days) and wound breaking strength (1258.33±59.74) when compared to nitrofurazone (16.36±0.08 days and 1683.33±20.28). Further, the results of the virtual screening revealed that among the three molecules tested, morusin showed minimum binding (-9.47 kJ mol-1) and docking energy (-11.35 kJ mol-1) when compared with the standard reference nitrofurazone (-6.03 kJ mol-1 and -6.29 kJ mol-1).
Conclusion: the active constituents isolated from *Morus laevigata* supports the wound healing ability of the different solvent extracts in terms of different binding models.
Keywords: Morusin; GSK3-β; in silico analysis; nitrofurazone, wound healing.

INTRODUCTION

Wound healing or wound repair is an intricate process in which the skin or organ or tissue repairs itself after injury [1]. Wound caused can be healed by a spontaneous process in an organism through a cascade of events, which starts by switching on various chemical signals in the body; this facilitates the restoration of anatomical continuity and function [2, 3]. The primary benefits of plant-derived medicines are that, they offer profound therapeutic benefits and more affordable treatment [4]. In drug discovery, the secondary metabolites like terpenoids, phenolics, alkaloids, saponins, etc., are of potential medicinal interest. Secondary metabolites are synthesized by the plant during development and are time, tissue and organ specific. They can be induced by biotic and abiotic factors. In contrast to primary metabolites, they are not present in all plant cells and not essential to sustain growth [5]. *Morus* plant species possess enormous importance in medicinal, economical, industrial, clinical, and domestic fields. *Morus laevigata*, a white variety, is a popular hybrid species which is highly appreciated for its delicious fruit, which is eaten fresh as well as in dried form and consumed in marmalades, juices, liquors, natural dyes and cosmetics industries [6]. Morusin, a major constituent profoundly found in the *Morus* species and in particular to *Morus laevigata*, leaves are rich in citrulline and hydroxyprolines and the seeds are the richest source of free amino acids [7].

Numerous studies have shown that the Wingless type (Wnt) signaling pathways plays a key role in various cellular functions including cell proliferation, differentiation, survival, apoptosis and migration [8,9]. Wnt signaling pathway has been divided into two sub-classes: the canonical pathway (Wnt/β-catenin pathway) and the noncanonical pathway. It has been proven that Wnt/β-catenin pathway can enhance wound healing [10] through the inhibition of GSK3-β, an important regulatory protein.

In-silico docking studies play an important role in the identification of lead molecules in drug designing and discovery. In the present study, an attempt was made to study the effect of different solvent extracts of *Morus laevigata* on wound healing activity. Further, different binding models were predicted against the target protein GSK3-β, to identify the correct binding geometry with various conformations in a non-covalent fashion at the active pocket [11] for each ligand molecule isolated from *Morus laevigata*.

MATERIALS AND METHODS:

Collection, extraction and Preliminary phytochemical analysis of Plant extracts: The plant material was collected from Mulberry Germplasm Center, Central Silk Board, Hosur, Tamil Nadu. Leaves were subjected for shade drying under laboratory conditions. The dried leaf material was powdered and subjected for hot soxhlet extraction utilizing petroleum ether, chloroform and methanol sequentially. The preliminary qualitative phytochemical studies were performed for all the solvent extracts to test the presence of chemical groups Viz. steroids, glycosides, terpenoids, saponins, alkaloids, flavonoids, tannins, carbohydrates, proteins and amino acids [12].

Animals: Albino rats (150-180g) of both sexes were used in the experiment, and they were housed under standard environmental conditions. All animal experiments were carried out in accordance with the guidelines of CPCSEA. The animal ethical committee approval was obtained to conduct the animal experiments (NCP/IAEC/CL/40/12/2011-12 dated 05-01-2012).

Drug formulation: The drugs were prepared for each of the extracts for topical administration. 5g of each extract was mixed with 45g of white petroleum jelly to get 10% (w/w) concentration and were applied topically in the form of ointments [13]. 0.2% (w/w) Nitrofurazone was used as a standard reference.

Wound healing activity: In the present study, the wound healing activity of *Morus laevigata* extracts was conducted in two models viz. excision and incision wound model. The plant extracts (10% w/w) were applied topically in the form of ointments. The animals were numbered, weighed and then divided into five groups with six animals in each group. Group 1 served as the control and received 5% white petroleum jelly, an ointment base. Group 2 served as the reference standard and rats were treated by Nitrofurazone ointment (0.2% w/w). Group 3, 4 and 5 were treated with petroleum ether, chloroform and methanolic extract of *Morus laevigata* respectively. The ointments were externally applied once a day, till the epithelialization was complete.

Wound breaking strength was measured by the tensiometer on the 11th day by adopting continuous constant water flow technique as described by lee. [14], the breaking strength was expressed as the minimum weight of water necessary to bring about gaping of the area. Readings were recorded for a given incision wound [15].

Statistical analysis: The observations are reported as mean ±SE. One-way ANOVA (analysis of variance); a statistical tool was used to measure the means between the groups. The results obtained were compared with the control group and the value $P < 0.05$ was considered as statistically significant.

In silico Studies: Auto Dock 4.2 was used for in-silico docking studies to determine the binding of drug molecules viz. morusin, citrulline and hydroxyproline which have been isolated from *Morus laevigata* [7]. Nitrofurazone, a standard drug and CHIR-98014, a positive control (ATP-competitive inhibitor) were selected to compare the binding results [16-18].

Tools and servers: Structures of drug molecules were drawn and analyzed using ChemDraw Ultra V6.0. The geometry optimization was done using chemsketch v12.01. 3D coordinates were prepared using openbabel. The protein structural file (PDB ID: 1Q5K) was fetched out from PDB (www.rcsb.org/pdb). Protein–ligand interactive visualization and analysis was carried out in Pymol viewer 1.5.4. ADME-T properties of molecules were calculated using Organic chemistry portal (http://www.organic-chemistry.org/prog), a web-based application for predicting in silico ADME-T

property. Molecular docking was performed using AutoDock v4.2 on a Lenovo G570 machine (Intel Core i5-560 M Processor 2.66 GHz, 4 GB memory) with Windows 7 operating system (64-bit).

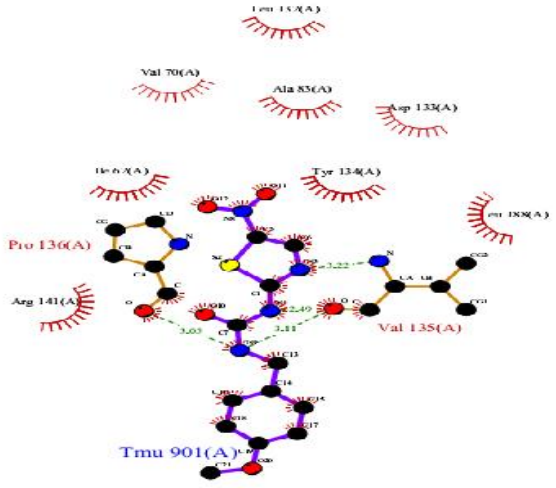
Primary screening of the drug molecule: The Lipinski rule-of-five also known as Pfizer's rule of five (RO5) was employed for the screening of competent drug molecules based on their molecular properties. Rule of five has become a computational approach for the estimation of solubility and permeability of new drug candidates. It is also a standard protocol to check the pharmacological and biological properties of the ligand for virtual screening (VS) [19]. The rule describes the molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion (ADME) which makes sense for drug-likeness prediction and oral bioavailability [20]. Molinspiration (http://www.molinspiration.com/ cgi-bin/properties), an online tool was used to obtain parameters such as Molecular Weight, CLogP, H-Bond Donor, H-Bond acceptor and Rotatable bonds.

In silico ADME-Toxicology studies: The ADME-T properties of each drug molecule were calculated by submitting the competent drug candidates to the organic chemistry portal (http://www.organic-chemistry.org/prog), a web-based application which also provided the information about solubility, permeability, and drug-likeness of each drug molecule.

Structure-based virtual screening: The initial stage of Structure-based virtual screening is the identification of potential ligand-binding pocket. Binding pocket (active site) is a small grove like structure consisting of hydrophilic amino acids which help in the interaction of drug with the target molecule [21]. The structure of the active pocket was predicted using ligplot and the residues forming the active pocket were identified (Fig 1). (Ile 62, Val 70, Ala 83, Leu 132, Asp 133, Tyr 134, Val 135, Pro 136, Arg 141, Leu 188).

Preparation of ligands: Structure of the drug molecules were drawn in ChemDraw Ultra 6.0 assigned with proper 2D orientation (ChemOffice package) (fig. 2) and the structure of each ligand was analyzed using Chem-3D Ultra 6.0 (ChemOffice package). 3-D geometrical optimization was done using Chemsketch v12.01 (ACD/labs). Openbabel, a standalone tool was used to obtain 3D coordinates for all the drug candidates.

Preparation of macromolecule: Glycogen synthase kinase 3-β (GSK 3- β) is a serine/threonine kinase that has been implicated in pathological conditions such as diabetes and Alzheimer's disease. The crystal structure was isolated in the presence of inhibitor TMU (N-(4-Methoxybenzyl)-N'-(5-Nitro-1,3-Thiazol-2-Yl)urea) [22]. The protein structure file (PDB ID: 1Q5K) was retrieved from PDB (www.rcsb.org/pdb) and is edited by removing the heteroatoms like metal ions, water and ligand molecule. Later it was added with C-terminal oxygen, polar hydrogen, and Gasteiger charges.



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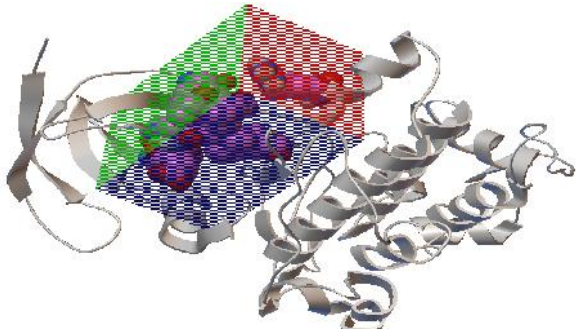

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B

Fig. 1: Binding pocket of GSK3-β as predicted by Ligplot (A) and grid box encompassing the active pocket with its 3-D coordinates (B)

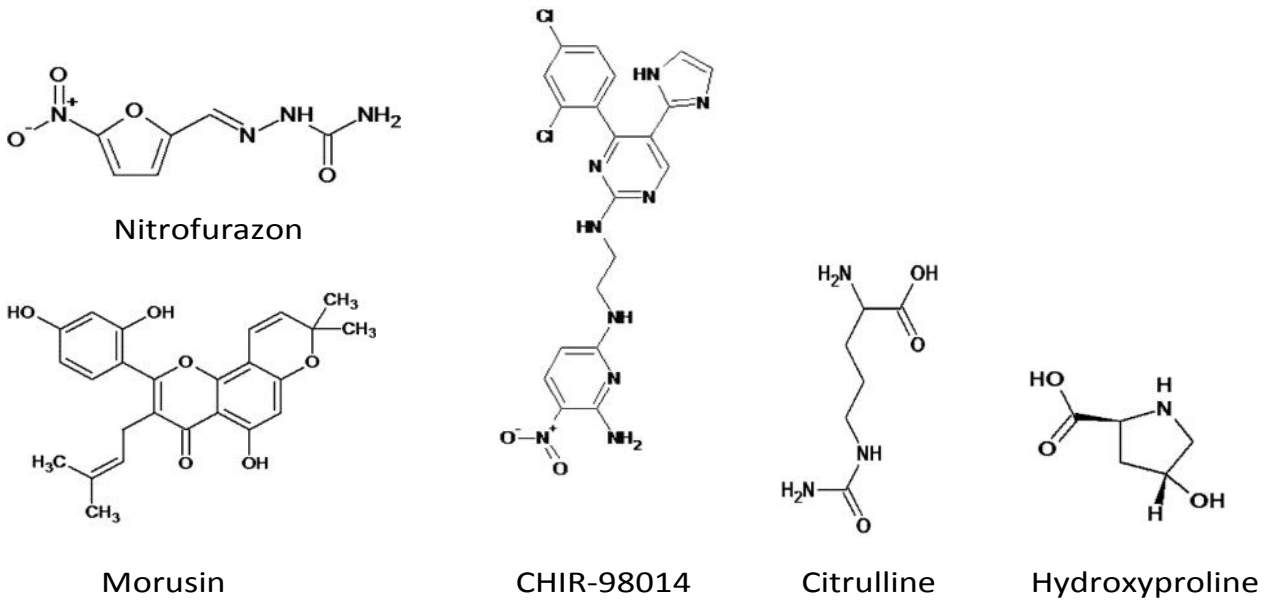


Fig 2: 2D Structure of ligand molecules

Molecular docking: Automated docking was used to study the binding of different drug molecules to the active pocket of GSK3-β. A genetic algorithm method implemented in the AutoDock 4.2 was employed to study appropriate binding modes of the ligand in different conformations. For the ligand molecules, Gasteigere–Marsili partial charges [23] were assigned and non-polar hydrogen was merged. All the torsions were allowed to rotate during docking. The grid map was set around the residues forming the active pocket, which was predicted using ligplot. Grid file was generated using AutoGrid program and Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for energy minimization using default parameters. Binding energy and docking energy were calculated using the following equation [24].

Docking energy =A+B+C+D

Where A=Intermolecular energy, B=Total internal energy, C=Torsional free energy and D=energy of the unbound system

Binding energy =A+C,

Where A=Intermolecular energy and C=Torsional free energy.

RESULTS:

Qualitative Phytochemical Analysis: The results of the qualitative phytochemical analysis of different leaf extracts of *Morus laevigata* revealed the presence of steroids, glycosides, terpenoids, saponins, alkaloids, flavonoids and tannins. The petroleum ether extract of Morus laevigata showed the presence of glycosides, flavonoids, tannins, carbohydrates. The chloroform extract showed the presence of steroids, glycosides, terpenoids, saponins, alkaloids, flavonoids, tannins, carbohydrates and methanol extract showed the presence of steroids, glycosides, terpenoids, saponins, alkaloids, flavonoids, tannins, carbohydrates, proteins and amino acids (Table. I)

Table I: Qualitative phytochemical analysis in different species of Mulberry

Tests	MLPE	MLCH	MLME
Steroids	-	+	+
Glycosides	+	+	+
Terpenoids	-	+	+
Saponins	-	+	+
Alkaloids	-	+	+
Flavonoids	+	-	+
Tannins	+	+	+
Carbohydrates	+	+	+
Proteins	-	-	+
Amino acids	-	-	+

Where:
MLPE: *Morus laevigata* petroleum ether extract,
MLCH: *Morus laevigata* chloroform extract,
MLME: *Morus laevigata* methanol extract

Acute toxicity studies:

The acute toxicity studies were carried out using albino mice as per staircase method and the mortality rates were observed after 48 hours [25]. Accordingly the LD50 was found to be 2 g/kg body weight for all the extracts.

Wound healing activity:

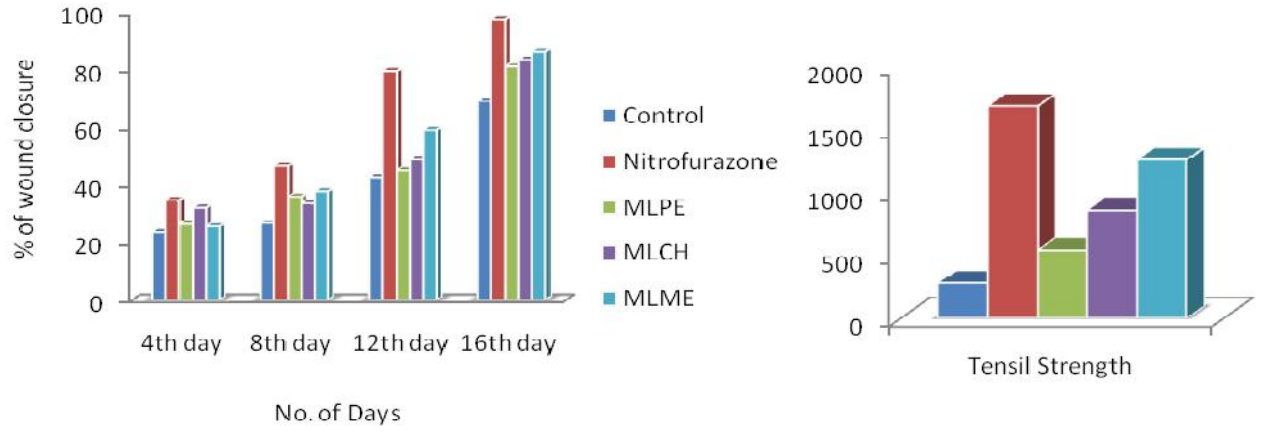
Excision wound model: In the present investigation, the methanol extract treated animals showed the significant promotion of wound healing in both the wound models viz., excision and incision. In excision wound model, the mean percentage closure of wound area was calculated on the 4, 8, 12 and 16th post wounding days respectively are shown in Table II and Fig. 3(A).

The epithelization of wound with 10% (w/w) petroleum ether, chloroform, and methanolic extract ointment treated group was found to be earlier as compared to control. In the 10% (w/w) methanolic extract ointment treated rat, the percentage of wound contraction was 86.5 ±1.57 on 16th day. And in the standard group, the wound contraction was 97±0.58% on day 16. In the 10% (w/w) chloroform extract ointment treated rats, the wound contraction was 83.67±2.16% on the 16th day, whereas in the 10% (w/w) petroleum ether extract ointment treated rats, the wound contraction was 81.33±1.15%. The wound healing was measured in all the groups till 16th day and further observations were done for the completion of epithelization.

Table II: Wound healing activity

Treatment	Percentage of wound closure (original wound area)				Mean time of epithelialization (days)	Tensile strength
	4th day	8th day	12th day	16th day		
Control	23.67±1.28	26.83±0.87	42.83±0.60	69.33±1.69	23.11±0.54	280.00±21.29
Nitrofurazone	34.83±1.68*	46.67±2.06*	79.67±1.17**	97.83±0.58**	16.36±0.08**	1683.33±20.28**
MLPE	26.50±0.76	36.00±1.24*	45.17±1.14	81.33±1.15	19.69±0.28*	540.00±13.90*
MLCH	32.17±1.35**	33.67±1.52	49.17±2.30*	83.67±2.16*	19.19±0.55*	856.67±17.64*
MLME	25.67±2.12*	37.83±1.01*	59.17±1.78*	86.50±1.57**	18.53±0.33**	1258.33±59.74*

Each value represents mean ±SEM; *=p<0.05, **=p<0.01; *n*=6 in each group



(A) (B)

Fig 3: percentage of wound closure (A) and Graphical representation of tensile strength (B)

The control group animals took a mean time of 23.11±0.54 days for epithelization whereas in standard group it was found to be 16.36±0.08 days. Methanol, chloroform and petroleum ether extracts took a mean time of 18.53±0.33, 19.19±0.55, and 19.69±0.28 days respectively for complete epithelization.

Incision wound model: The breaking strength of the skin in incision wounds was increased in extract treated groups (methanol, chloroform extract and petroleum ether) to a significant extent, i.e., 1258.33±59.74, 856.67±17.64 and 540±13.90 respectively. In the control, the tensile strength was observed to be 280±21.29. The results were comparable to the standard drug, nitrofurazone that was applied topically every day (1683.33±20.28) along with all the extracts (Table II and Fig. 3(B)).

Primary screening of the drug molecule: Primary screening was done to assess the drugability of the selected molecules. Lipinski rule-of-five (RO5) was employed to identify the suitable drug candidates based on their molecular and structural properties. Three compounds viz. morusin, citrulline and hydroxyproline were selected based on their molecular weight, ClogP value, hydrogen bond donors, hydrogen bond acceptors and presence on rotatable hydrogen bonds. The results are shown in Table III.

***In silico* ADME-Toxicology studies:**

Characterization of absorption, distribution, metabolism, excretion and toxicity studies is a crucial step in high throughput drug discovery. Hence, based on the primary screening, selected drug candidates were examined for mutagenicity, tumorogenicity, irritation and reproductive effects and the results are expressed in terms of high, moderate and nil risk factors. Based on ADMET properties along with their solubility, drug likeliness and respective drug scores were measured. The results are shown in Table IV.

Molecular docking: In the present work automated docking was used to assess the orientation of drugs bound in the active pockets of GSK3-b. AutoDock 4.2, a script driven flexible automated and random search docking technique operates by altering the ligand with several rotatable bonds to predict the binding interaction between target receptor. Lamarckian genetic algorithm method was implemented in the current study. The results of the molecular docking studies are shown in table V and the graphical representation of all the drugs with their binding energies are shown in Fig 4. Among the five compounds studied, Morusin showed better docking energy of -11.35 kJ mol-1 with the intermolecular energy of -10.36 kJ mol-1 and two hydrogen bonds when compared to the standard drug nitrofurazone, which has the

Table III: Prediction of Drug- likeliness (RO5)

Compound	Molecular Weight [g/mol]	cLogP	H-Bond Donor (less than 5)	H-Bond Acceptor (less than 10)	Rotatable Bond (less than 10)
Nittrofurazone	198.13624	0.2	2	5	2
Chir-98014	486.31408	4.2	3	9	7
Morusin	420.45446	5.5	3	6	3
Citrulline	175.18572	-4.3	4	4	5
Hydroxyproline	131.12986	-3.3	3	4	1

Table IV: In silico ADME-T studies

Compound	Mutagenic	Tumerogenic	Irritation	Reproductive effect	Solubility	Drug-likeliness	Drug score
Nittrofurazone*	H	N	N	M	-3.34	0.01	0.32
Chir-98014*	N	N	N	N	-8.38	-1.31	0.24
Morusin	N	N	N	N	-4.94	-0.78	0.29
Citrulline	N	N	N	N	-0.98	-12.6	0.49
Hydroxyproline	N	N	N	N	-0.31	0.91	0.85

N -No risk, M -Medium risk, H -High risk, * -Standard antibiotics

Table V: Molecular docking result

Compound	Docking energy (kJ mol^{-1})	Binding energy (kJ mol^{-1})	Intermol energy (kJ mol^{-1})	Total internal energy	Torsional energy (kJ mol^{-1})	Energy of the unbound system	Ligand efficiency	RMS	H-bonds	Bonding	Bond length	Bond energy
Nittrofurazone	-6.29	-6.03	-6.92	-0.13	0.89	-0.13	-0.43	0.0	4	1Q5K:A:ARG144:HH22::NITROFURAZOL:LIG1:O 1Q5K:A:ARG144:HH12::NITROFURAZOL:LIG1:O 1Q5K:A:ARG141:HH11:NITROFURAZOL:LIG1:O 1Q5K:A:VAL135:O::NITROFURAZOL:LIG1:H	1.782 1.767 2.168 1.808	-7.583 -7.707 -3.591 -7.897
Chir-98014	-7.75	-6.06	-8.75	-0.84	2.68	-0.84	-0.18	0.0	3	1Q5K:A:ASP260:OD2::CHIR_98014:LIG1:H 1Q5K:A:GLU268:OD2::CHIR_98014:LIG1:HN 1Q5K:B:TYR222:HH::CHIR_98014:LIG1:O	1.751 2.234 1.754	-1.076 -2.14 -0.786
Morusin	-11.35	-9.47	-10.36	-0.94	0.89	-0.94	-0.31	0.0	2	1Q5K:A:VAL135:O::MORUSIN:LIG1:H 1Q5K:A:VAL135:HN::MORUSIN:LIG1:O	2.113 2.017	-3.647 -2.243
Citrulline	-9.14	-5.42	-7.51	-1.86	2.09	-1.86	-0.45	0.0	3	1Q5K:A:ASP260:O::CITRULLINE:LIG1:H 1Q5K:A:CYS218:O::CITRULLINE:LIG1:H1 1Q5K:B:ASP260:O::CITRULLINE:LIG1:H2	1.95 2.094 1.991	-0.719 -1.94 -1.477
Hydroxyproline	-6.65	-3.85	-4.74	-1.4	0.89	-1.4	-0.43	0.0	1	1Q5K:B:GLN185:OE1::HYDROXYPROLINE:A:HYP1:HD	1.736	-0.809

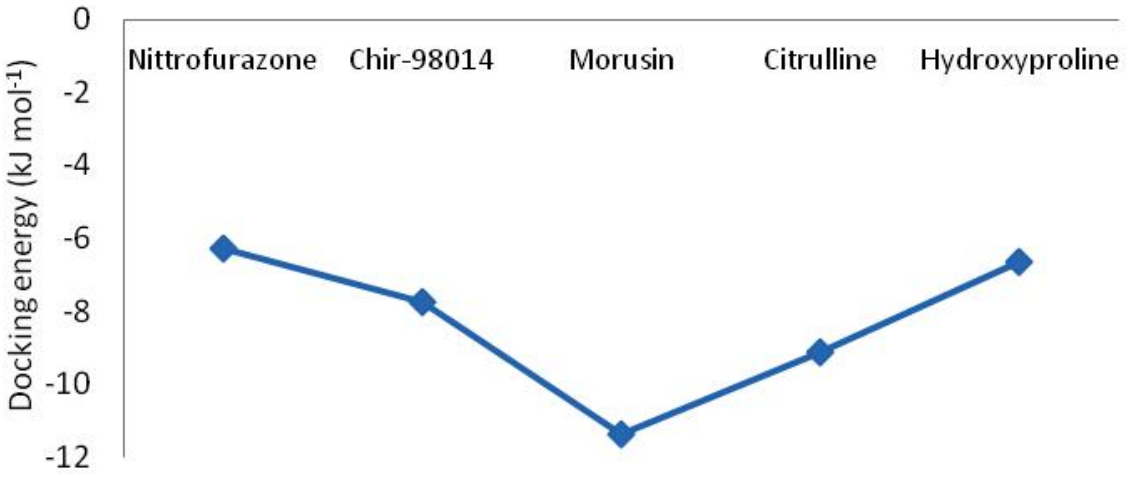
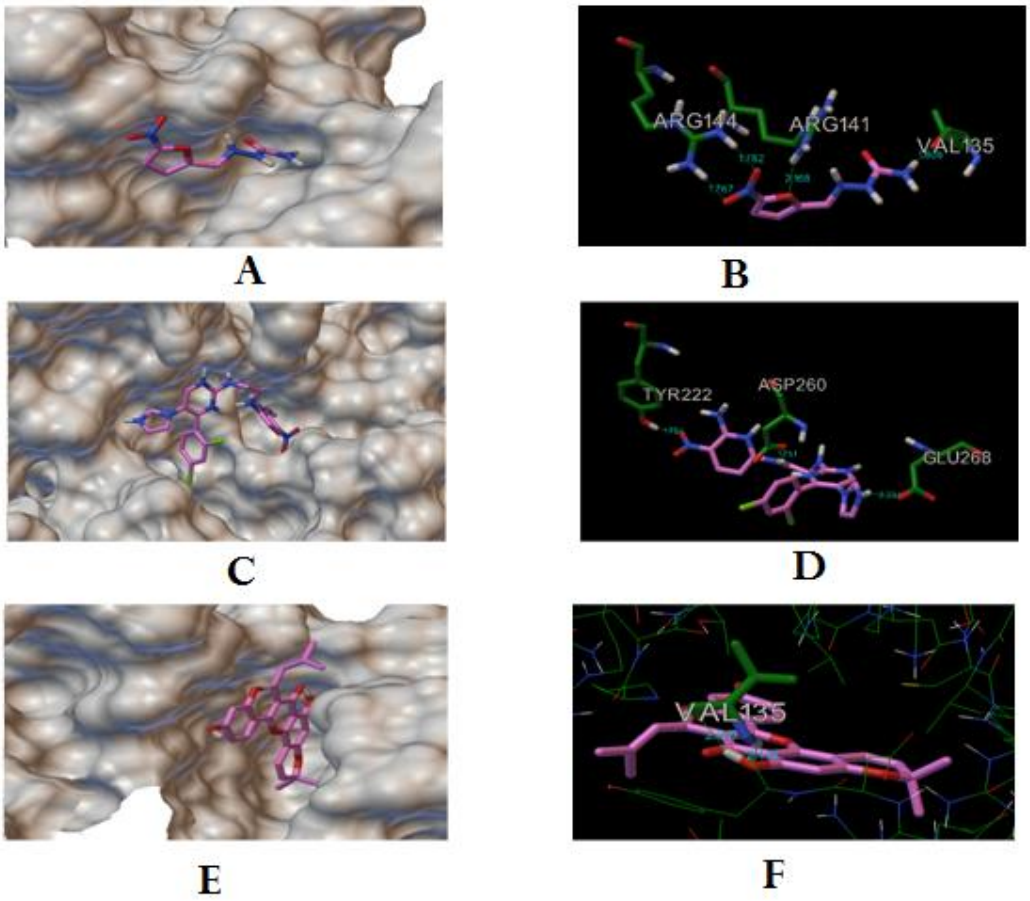
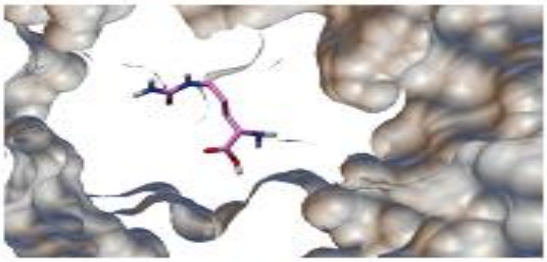


Fig 4: docking energy of drug molecules

Fig 5: Molecular interactions showing the binding and hydrogen bond formation of Nitrofurazone (A),(B), Chir-98014 (C),(D), Morusin (E),(F), Citrullin (G),(H) and Hydroxyproline (I),(J) with the active pocket of GSK 3-β





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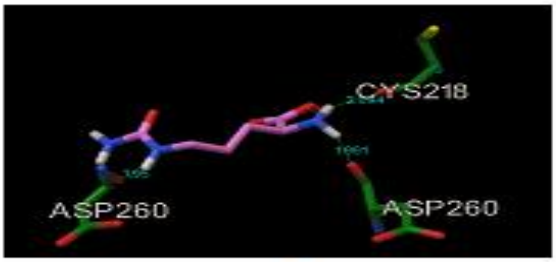

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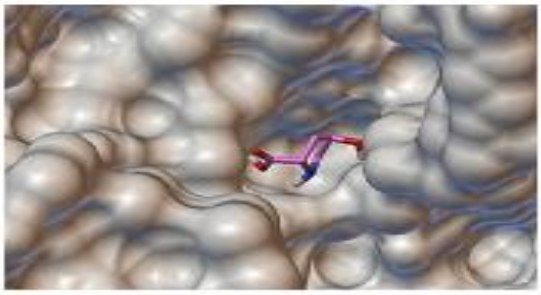

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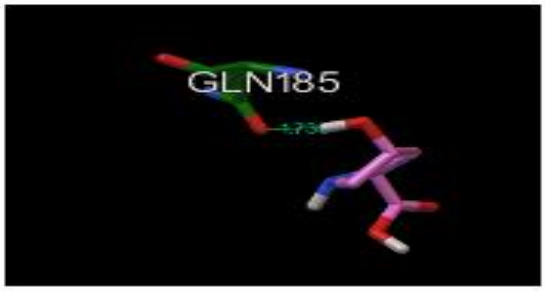

J

docking energy of -6.29 kJ mol-1 and intermolecular energy of -6.92 kJ mol-1 with four hydrogen bonds. Further, the positive control CHIR-98014 showed the docking energy of -7.75 kJ mol-1 and intermolecular energy of -8.75 kJ mol-1 with three hydrogen bonds. Citrullin and hydroxyproline also showed better interactions with GSK3-β with the docking energies of -9.14 kJ mol-1 and -6.65 kJ mol-1 and intermolecular energies of -7.51 kJ mol-1 and -4.74 kJ mol-1 with three and single hydrogen bonds respectively. Molecular interactions between the drug and the protein molecule are shown in the Fig. 5.

DISCUSSION:

Phytochemical screening revealed the presence of several bioactive compounds viz. tannins, flavonoids, alkaloids, proteins and other important constituents. The plant is a very good source of ascorbic acid, of which over 90% is present in a reduced form, and also contains carotene, Vitamin B1, folic acid, folinic acid, isoquercetin, quercetin, tannins, flavonoids and saponins, which act as a good source of natural antioxidants [26]. Flavonoids have been documented to possess potent antimicrobial, antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing [27-29]. There are reports that the plants having antioxidant property would also enhance wound-healing activity [30]. Similarly, several studies have also reported that triterpenoids are known to promote the wound-healing process, mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization [31]. These active constituents promote the process of wound healing by increasing the viability of collagen fibrils, by increasing the strength of collagen fibers either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis [32].

The constituents of the *Morus laevigata* extracts include several phenolic compounds; primarily the flavonoids confirmed by the qualitative chemical tests. Recent studies have shown that constituents like flavonoids and other phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation and antimicrobial activities. The phytoconstituents present in Morus species, which either due to their individual or additive effect fastens the process of wound healing [33-35] could be the reason for wound healing activity of Morus laevigata.

The discovery of drugs can be accelerated by the use of computational methods in lead identification and optimization [36]. Virtual screening helps in identifying,

screening and selection of such potent drug molecule [37,38]. Evidences have suggested that Wnt signalling and its effector β-catenin play an important role in the wound healing process. Further, the down-regulation of elite target molecule GSK3-β, was found to disturb the Wnt signaling pathway enhancing wound healing activity [39, 40]. Several in silico studies have also been carried out on GSK3-β to study the binding models of different drug molecules. Hydroxyproline, one of the lead molecules in the current investigation was also found to be a specific marker of collagen and an important component of the extracellular granulation tissue matrix influencing rapid collagen turnover and accumulation. Hydroxyproline also helps in promoting the cutaneous wound healing through the elicitation of β-catenin-dependant Wnt pathway through the inhibition of GSK3-β, explaining the increased rate of wound contraction [41, 42].

CONCLUSION:

Determination of the various individual components of the phases of healing can provide important insights about events operative during wound repair. The results of the present study revealed that, among the three solvent extracts of *Morus laevigata*, methanolic extract showed significant wound healing activity than chloroform and petroleum ether extracts. The results of in silico binding studies showed encouraging results in terms of binding and docking energies. Theoretically, all the ligand molecules have bound to GSK3-β with specific orientation encompassing the active pocket. Among the tested molecules, docking of GSK3-β with morusin showed minimum binding and docking energy when compared to rest of the molecule and thus it can be considered as a good inhibitor of GSK3-β. Thus, the present study underlies the potent wound healing activity of the phytoconstituents present in Morus laevigata, thereby justifying its use in the indigenous system of medicine.

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