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Molecular docking and evaluation of wound healing activity of stigmastatone isolated from bark of *Celastrus paniculatus* Willd.

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Abstract

Glycogen Synthase Kinase3-β (GSK3-β) in various organisms have revealed physiological roles for the enzyme in differentiation and cell fate determination. The molecular docking of GSK3-β with stigmastatone isolated from the petroleum ether extract of bark showed the inhibition constant 1.21 nM whereas, standard drug nitrofurazone showed inhibition constant of 1.35 nM. Both extract and isolated constituent were studied for their potency using excision, incision and dead space wound models in rats. In stigmastatone treated animals, epithelialization was faster with 97.01% wound contraction on 18th post wounding day. Tensile strength of incision wound was significantly increased to 592.25±1.09g after intraperitoneal administration of stigmastatone (12 mgkg^{-1}). In dead space wound, a significant increase in weight, tensile strength and increased collagenation of granuloma tissue was observed.

Key words: Bark, *Celastrus paniculatus,* Docking, GSK3-β, stigmastatone, wound healing.

Introduction

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in drug discovery and development. Despite changing strategies in natural product research, which included sample selection and collection, isolation techniques, structure elucidation of the isolates, biological evaluation, biosynthesis have been increased, so the rate of discovery of truly novel natural product drugs were also increased. The advent of bioinformatics reduced the cost and time of drug screening process. In an effort to reduce the cost of developing new medicines and their time to market, pharmaceutical companies have attempted to streamline the drug discovery process using computational methods. Today, virtually every drug company of appreciable size has adopted computational methodology in most stages of the design process (Jorgensen 2004; Barril and Soliva 2006; Tramontano 2006). Many computational methods complement one another and may be combined to help rationalize the drug discovery process. In cases where it is possible to determine the 3-dimensional structure of the biomolecular target, molecular docking (Brooijmans and Kuntz 2003; Sousa et al. 2006) becomes possible and allows structure-based hit identification and/or lead optimization (Shoichet et al. 2002; Kitchen et al. 2004).

The role of Wnt signal transduction during wound healing remains unexplored. Wnts constitute a family of secreted glycoproteins with distinct expression patterns that regulate cell proliferation, migration and specification of cell fate in the embryo and adult organism (Veeman 2003). Wnts appear to be involved in differentiation processes by controlling polarity of cell division, cell growth and cell fate (Clark 1991). The role of Wnt signal transduction during wound healing remains unexplored. However, it is clear that the Wnt signaling pathway can play an important role in the skin. Genes encoding Wnts and other components of the pathway are expressed in skin during epithelialization and collagen matrix formation. Expression of those genes involved in the inhibition of cell signaling, activation of cell cycle, avoid the ubiqutinization of transcription factors (β- catanin) and signaling inhibitors (Fathke et al. 2004).

Wound healing is a highly ordered and well coordinated process that involves inflammation, cell proliferation, matrix deposition, tissue remodeling, collagenation and epithelialization. Many investigators evaluated the wound healing properties of many of the medicinal herbs, clinically on animal models using excision, incision and dead space models (Kumara Swamy et al. 2007; Sharath et al. 2010). In this paper an attempt has been made to screen the wound healing property of the constituent stigmastatone isolated from the bark of *Celastrus paniculatus* Willd. *in vivo* on Swiss albino rats employing excision, incision and dead space models. The mode of action of the stigmastatone molecule was hypothesized *in silico* by docking the molecule to GSK3-β protein an important regulatory enzyme whose inhibition promotes wound healing through β-catanin dependent Wnt signalling pathway.

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Materials and Methods

Plant material

Bark of *Celastrus paniculatus* were collected from the Bhadra Wild Life sanctuary of the Western Ghats region of Karnataka, India. The taxonomic identity was confirmed by comparing with the authenticated specimen deposited at Kuvempu University Herbaria (voucher specimen FDD No. 140). The bark was chapped and shade-dried for ten days and mechanically ground to a powder. The successive Soxhlet extraction was done using petroleum ether (40-60^{o}C), chloroform (60-80^{o}C) and methanol (60-80^{o}C) as solvents. The extract was filtered, pooled and the solvent was removed under reduced pressure at 40 ± 5°C using a rotary flash evaporator (Büchi, Flawil, Switzerland); the yield was 10g/200g bark powder.

Molecular docking

Automated docking was used to determine the orientation of inhibitors bound in the active site of GSK3-β. A genetic algorithm method, implemented in the program AutoDock 3.0, was employed (Bhat et al. 2003). The ligand molecules, stigmastatone and nitrofurazone were designed and the structure was analyzed by using ChemDraw Ultra 6.0. 3D coordinates were prepared using PRODRG server (Ghose and Crippen 1987) and preADMET server was used for drug likeliness prediction. The protein structure file 1Q5K was taken from PDB (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C terminal oxygen (Binkowski et al. 2003). For docking calculations, Gasteigere- Marsili partial charges (Gasteiger and Marsili 1980) were assigned to the ligands and nonpolar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map, which was centered at the following residues of the protein (Val 61, Ile 62, Asn64, Gly65, Ser66, Phe67, Gly68, Val 70, Lys 85, Leu 132, Val 135, Pro 136, Asp181, and Asp 200) were predicted from the CASTp server (Reya and Clevers 2005) were generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters. The number of docking runs was 50, the population in the genetic algorithm was 250, the number of energy evaluations was 100,000, and the maximum number of iterations 10,000.

Drug formulations

Two types of drug formulations were prepared for the compound stigmastatone. For topical administration 100 mg of stigmastatone was prepared in 20% sodium alginate to get 0.2% w/v gel. For oral administration, a suspension of 10mg/ml isolated compound stigmastatone was incorporated with 1% w/v gum tragacanth. The drug formulations were prepared every third day and the drug was administered orally by a feeding tube. The study was permitted by the Institutional Animal Ethical Committee (Reg. No. 144/ 1999/CPCSEA/SMG).

Wound healing activity

Four groups of animals containing six each were used for each of the excision and incision wound models. The animals of group I were considered as the control, the animals of group II served as the reference standard and treated with 0.2% w/w nitrofurazone (Furacin, Smithklin Beecham, Bombay, India) ointment. The animals of group III were treated with the isolated constituent stigmastatone and animals of group IV were treated with the petroleum ether extract of bark of *C. paniculatus*. Wound healing activity of the test drug stigmastatone was evaluated concomitantly following the methods of Morton and Malone (1972), Ehrlich and Hunt (1968) and Lee and Tong (1968) for excision, incision and dead space wound models respectively. The results of the *in vivo* experiments are expressed as mean±SE of six animals in each group. The data were evaluated by one-way ANOVA followed by Tukey's pair-wise comparison test. The values of $p>0.01$ and $p>0.05$ were considered as statistically significant.

Result and Discussion

The crude petroleum ether extract of stem was subjected to gradient polarity column chromatography using petroleum ether/chloroform as the eluent. Six fractions were collected; of which fraction3 contain the major compound with a few minor constituents. The vials of fraction3 were combined and then subjected to another column chromatography using petroleum ether/chloroform (9:1) as the eluent. Three 30 ml fractions were collected; the fraction 1a containing the single compound was confirmed by TLC showing single spot (Spraying reagent; vanillin-sulphuric acid, heated at 110°C). The compound obtained was a white powder with a melting point of 205°c-210°c. The compound was subjected to spectroscopic characterization include, IR (KBr): 3393.14cm-1 (br, OH), 2916.73 cm-1, 2872.48 cm-1 (C-H str. in CH3 and CH2) 1640.16 cm-1 (C=C str.), 1457.92 cm-1 (C-H deformation in CH2/CH3). ^{1}H NMR: (CDCl3) 5.72 (br. s, H-C(4)); 0.71 (s, 3 H-C(18)); 1.18 (s, 3 HC(19)); 0.96 *(d J* = 6.8, 3 H-C(21)); 1.61 rI.66, 1.721 (br. s, 3 H-C(26), 2.01 (*4, J* = 7.4, 2 H-C(28)); 0.94 *(1, J* = 7.4,3 HC(29)); 1.50,2.00, 2.40 (2 H-C(2)); 2.35 (2 H-C(6)); 1.00, 1.90 (H,-C(7), Hp-C(7)); 1.50 (H-C(S)); 0.90 (H-C(9)); 1.45 (2 H-C(l1)); 1.15, 2.05 (H,-C(12), HrC(12)); 1.05 (H-C(14)); 1.10, 1.60 (H,-C(15), HgC(15)); 1.25, 1.90 (Hz-C(16), HrC(16)); 1.05 (H-C(l7)); 1.40 (H-C(20)); 1.10, 1.40 (2 HC(22)); 1.25 (2 H-C(23)).

Members of the Wnt family are secreted glycoproteins that regulate cell proliferation, migration, and specification of cell fate in the embryo and adults (Veeman et al. 2003). Wnt proteins are classified according to their ability to promote stabilization of b-catenin in the cytoplasm. The b-catenin dependent Wnt pathway signals through cytoplasmic stabilization and accumulation of b-catenin in the nucleus for the expression of target genes. It has been reported that prolonged activation of the b-catenin-dependent pathway resulted in the partial regeneration of epithelial appendages in the wound. Promotion of Wnt signaling by Lithium Chloride that is a selective inhibitor of GSK3-b or the prolonged expression of Wnt-5a promotes partial regeneration of epithelial appendages in adult murine skin and demonstrates the plasticity of adult epithelial cells during wound-healing process (Fathke et al. 2006).

In the present study the comparative docking of isolated constituent stigmastatone and standard drug nitrofurazone was done against GSK3-β. The ligands-binding pocket of GSK3-β is composed of two regions, namely the A and B regions (Bhat et al. 2003). The stigmastatone was bound to GSK3-β (Fig 1a) The A region is the bottom of the pocket and offers an essential hydrogen bond donor (the backbone NH of Val135) which forms a hydrogen bond with the oxygen atom of the stigmastatone with the bond distance 2.11Å (Fig 1b). The minimum docked energy of stigmastatone is -8.78kcal/mol. The docking result of the standard drug nitrofurazone (Fig. 1c) was as follows, docking energy,-6.37kcal/mol, inhibition constant, 1.35nM and intermolecular energy -6.28 kcal/mol. The accuracy of dockings was evaluated by the root-mean square deviation (RMSD) of docked ligand from original crystal structure.

RMSD values for stigmastatone is 0.8 whereas, the nitrofurazone was 1.1. Lithium chloride is a selective inhibitor of the key

regulatory enzyme GSK3-b that regulates the expression and accumulation of b-catenin and promotes the woundhealing process. Several GSK3-b inhibitors have been described and most of the observed effects are in vitro and cellular studies. Among these, inhibitors are paullones (Leost et al. 2000), thiadiazolidinones (Martinez et al. 2002), lupeol (Harish et al. 2008). Hence, GSK3-b was selected as target for the docking studies. GSK3 belongs to the superfamily of mitogen-activated protein (MAP) kinases.

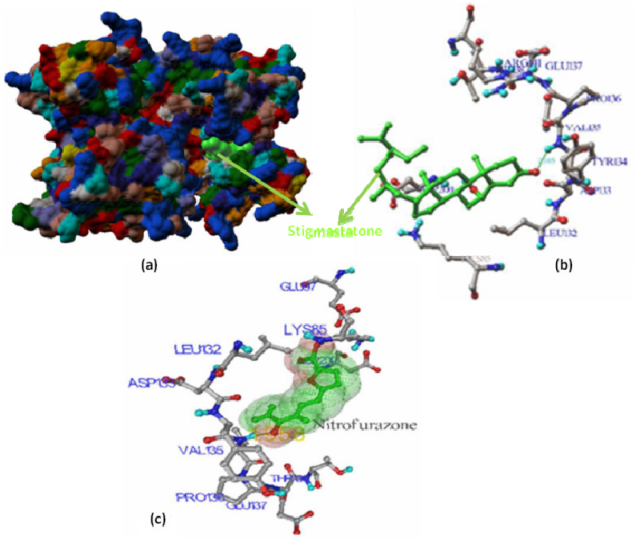

Figure 1: a) The stigmastatone was bound to GSK3-β. b) A hydrogen bond formed between oxygen atom of the stigmastatone and Amide nitrogen of Val135 of GSK3-β. c) The docking result of the standard drug nitrofurazone.

The molecular docking studies revealed that the isolated constituent stigmastatone showed very good binding activity towards glycogen synthase kinase 3-β by calculating binding energy, docking energy, inhibition constant, intermolecular energy etc. On the basis of these calculations the isolated constituent and their extracts were screened for wound healing activity by employing excision, incision and dead space models.

fibroblastic stage whereby the area of the wound undergoes shrinkage. It has three phases, inflammatory, proliferative, and maturational, and is dependent upon the type and extent of damage, the general state of the host's health, and the ability of the tissue to repair. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis, and collagen deposition in the proliferative phase. In maturational phase, the final phase of wound healing the wound undergo contraction resulting in a smaller amount of apparent scar tissue.

The studies on excision wound-healing model showed that there is almost complete healing on the 18th post wounding day with petroleum ether extract and its constituent stigmastatone. The petroleum ether extract-treated animals showed significant reduction in the wound area (93.51 %), faster rate of epithelialization (19.7±0.14). In control animals, the duration of healing was extended up to 22 days. After 18th day of post wounding, the epithelialization was complete in animals treated with the standard drug Nitrofurazone, with 96.94 % of wound contraction. Stigmastatone showed significant reduction in the wound area (97.1 %). The scar area and time required in days for complete epithelialization of excision wound was also significant (18.5±0.12). Table 1 shows the period of epithelialization and percentage of wound contraction due to the effect of petroleum ether extract and stigmastatone. In excision wound model, significant wound-healing activity was observed in the animals treated with petroleum ether extract and the stigmastatone, and there is a 99 % significant (P>0.01) decrease in the period of epithelialization and increase in wound contraction rate observed in those groups of animals (df = 6) when compared to control. In stigmastatone and the standard reference drug nitrofurazone-treated animals, epithelialization was completed on 18th post wounding day. The breaking strength is the strength of a healing wound and is measured experimentally by the amount of force required to disrupt it. In the beginning a wound will be having little breaking strength because the clot alone will be holding the edges together. Thereafter, breaking strength increases rapidly as collagen deposition increases and crosslinkages are formed between the collagen fibers.

Table 1: Effect of topical application of petroleum ether bark extracts of *Celastrus paniculatus* and its isolated constituent stigmastatone on excision wound model.

Treatment	Percentage of closure of excision wound area (original wound area 250mm2)				Epithelialization in days
	Day4	Day8	Day 12	Day16	
Control	246.3±0.09	193.3±0.11	114.2±0.97	67.3±0.62	22.50±0.40
Nitrofurazone	244.5±0.09	187.5±0.30	94.8±0.80	43.9±0.65	18.2±0.22**
Stigmastatone	245.4±0.22	190.5±0.32	101.9±0.85	63.7±2.87	18.5±0.12*
Pet ether extract	245.3±0.10*	192.2±1.06	104.0±0.65	63.3±2.35	19.7±0.14*
F-Value	42. 0	87.4	20.0	67.4	75.43

Values are mean ± SE; n=6 in each group. **-significant at $P<0.01$ and *-significant at $P<0.05$ are compared to control.

Table 2 depicts the wound-healing effect of petroleum ether extract and the constituent stigmastatone in the incision wound model. The petroleum ether extract showed significant increase in the tensile strength on 10th post wounding day (442.8±10.2g). Significant increase in skin breaking strength was noticed in the animals treated with stigmastatone (537.4±3.01g). The animals treated with standard reference drug Nitrofurazone showed significant increase in the tensile strength of the incision wound (584.8±5.0g).

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the

Table 2: Effect of oral administration of stigmastatone and petroleum ether extract of *C. paniculatus* on Incision wound model.

Group (n)	Breaking strength (g)
Control	351.7±1.3
Nitrofurazone	584.8±5.0**
Stigmastatone	537.4±3.01
Petroleum ether extract	442.8±10.2*
F-Value	98.4

Values are mean ± SE; n=6 in each group.
**-significant at $P<0.01$ and *-significant at $P<0.05$ are compared to control.

In dead space wound model, the petroleum ether extract exhibited highest healing property as it is revealed by the significantly increased dry-weight of the granuloma tissue (20.5±0.8mg),

increased tissue breaking strength (460.2±9.7g). In stigmastatone treated animals significant increase in the dry weight of the granuloma tissue (21.4±0.31mg) with increased tissue breaking strength (512.2±10.8g), was observed. The data is depicted in Table 3.

Table 3: Effect of oral administration of isolated constituent stigmastatone and petroleum ether extract of *Celastrus paniculatus* on Dead space wound model.

Treatment	Granulation Tissue dry weight (mg/100g)	Breaking strength (g)
Control	11.8±0.79	369.8±2.0
Stigmastatone	21.4±0.31	512.2±10.8
Petroleum ether	20.5±0.8	460.2±9.7
F-Value	82.3	77.4

Values are mean ± SE; n=6 in each group.
**significant at $P<0.01$ and *significant at $P<0.05$ are compared to control.

There are reports that the plants having antioxidant property would also enhance wound-healing activity (Shirwaikar et al. 2003). Similarly other studies 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. 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 (Getie et al. 2002).

Conclusion

Stigmastatone has been proved to be one of the potent wound healing agent which has been shown to elicit the cutaneous wound healing better than the reference drug nitrofurazone. By *in silico* analysis, stigmastatone molecule is promoting the cutaneous wound healing through the elicitation of beta catanin dependant wnt pathway through the inhibition of GSK3beta.

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