Chapter -5

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Wounds, if untreated can become chronic. Chronic wounds can seriously affect the quality of life in patients. Currently available wound healing agents are often expensive and associated with toxicity (Guo et al 2010). Plants and their extracts have immense potential for management and treatment of wounds. The phytomedicines for wound healing are not only cheap and affordable but are also safe (Singh et al 2014). Fruit of *Barringtonia acutangula* is used traditionally in western Odisha as wound healing agents. However, there is no scientific evidence, so the present work is undertaken to investigate the wound healing activity of fruits of *Barringtonia acutangula*.

Detailed pharmacognostical study of a plant drug is very necessary before its use in the field of research and also in pharmaceutical formulation. It also helps to identify other allied species and adulterants from the authentic drug. The standardization on pharmacognostic parameters of a crude drug is an integral part to establish its correct identity. The pharmacognostic parameters and standards must be established before introduction of any crude drug in Herbal Pharmacopoeia (Verma et al 2008). To fulfil the above requirement, the pharmacognostical studies of fruit and seed of *Barringtonia acutangula* were undertaken.

Morphological evaluation of crude drug plays an important role in identification and detection of adulteration. It is the simplest technique to check the quality of crude drug as well as quickest means to establish the identity and purity of particular drug (Rungsung et al 2014). The longitudinal wrinkled surface of fruit and presence of longitudinal lines on seed are important characteristic features for identifying the plant. A research work cannot be considered scientific unless the test sample is not standardized. Therefore, microscopic and macroscopic techniques were used to evaluate the morphology of fruits of *Barringtonia acutangula*.

Microscopic approach utilizes techniques such as light microscopy to analyze characteristics such as the presence or absence of particular cell types to distinguish between the desired plant species and plant part at ultrastructural level (Subramanion et
al 2012). Many patches of vascular strands, stone cells of different shape and sizes in mesocarp region and the presence of oleoresin canals and numerous starch grains in the endosperm were found helpful in the identification of this plant. The calcium oxalate crystals in higher plant are the mineral deposits, which are formed by the combination of environmentally derived calcium and biologically synthesized oxalate, and may be deposited within intravacuolar chambers of specialized cells in any tissue (Nakata et al 2003). The presence of prismatic calcium oxalate crystals is one of the important parameter for identification of *Barringtonia acutangula*. Presence of highly lignified sclereids, starch granules, vessels of different types and calcium oxalate crystals are the important diagnostic characters from powder analysis. Different cellular component like lignin, starch materials and phenolic compounds were identified histologically by reaction with reagents and chemicals at different zones.

Fluorescence study is considered for first line standardization of crude drug. Light of short wave length is very active in producing fluorescence and for this reason ultraviolet light produces fluorescence in many substances which do not visibly fluorescence in day light (Arya et al 2011). If the substances themselves are not fluorescent, they may be converted into fluorescent derivatives or decomposition products by using different reagents. Hence, the crude drugs are often evaluated qualitatively in this way and it serves as an important parameter for pharmacognostic evaluation of crude drugs (Zhao et al 2011). The different colour produced by powdered drug under ultraviolet light may be one of the criteria for identification. Powdered crude drug showed different colours at ordinary day light when it was treated with different chemical reagents.

Physico-chemical evaluation of crude drugs also ensures the identity of drug and determines the quality and purity of drugs. The evaluation of crude drug is necessary as biochemical variation takes place in drug during treatment with other chemicals, storage for long time, adulterated with sub-standard drugs (Jarald et al 2007). Total ash is the important standardization parameter for checking the purity of the drugs by identifying the presence or absence of foreign inorganic matter such as metallic salts or silica (Anonymous 1986). It indicates presence of various impurities like carbonate, oxalate and silicate. The water soluble ash is used to estimate the amount
of inorganic compound present in drugs. The acid insoluble ash indicates the presence of earthy material in drug (Tatiya et al 2012). The total ash, water soluble ash and acid insoluble ash were found more in fruit than seed.

Moisture content of drugs should be at minimal level to discourage the growth of bacteria, yeast or fungi during storage (Tatiya et al 2012). The moisture content of the drug is not too high thus it can prevent bacteria, fungi or yeast growth, as the general requirement for moisture content in crude drug is not more than 14% w/w (Anonymous 1986).

The extractive values determine the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytocomstituents. The compositions of these phytocomstituents depend upon the nature of the drug and the solvent used. It also gives an indication whether the crude drug is exhausted or genuine (Tatiya et al 2012). Water and methanol extractive value was found more in both fruit and seed. So, we attempt to extract the dried fruit with methanol and water. It was expected that polar phytocompounds such as polyphenolic compounds and flavonoids can be extracted from *Barringtonia acutangula* fruit.

The local people in western Odisha directly used crushed ripe fruit on the wound surface. The extract in the form of ointment were evaluated for wound healing activity in normal rats, in rat with infected wound and diabetic rats. Both excision and incision models were used. Parameters like wound contraction, tensile strength, hydroxyproline content were studied. Histopathology of wounded areas was also done.

Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal stage and wound contraction is a process of shrinkage of area of the wound (Greenhalgh et al 2003; Lakshmi et al 2011). There was significant wound contraction by both methanolic and aqueous extracts of *Barringtonia acutangula* fruit. In histopathological studies, a greater degree of epithelialization, collagen and fibroblastic deposition was observed in both methanolic and aqueous fruit extract treated rats. In the control group wounded area skin shows ulceration with inflammation exudates. There is mild increase in fibrous tissue as well as granulation tissue and
congested blood vessels. Wound healing is incomplete. In 20%w/w methanolic and aqueous extract treated rats intact portion of skin reveals normal histology.

Wound healing is characterised by neovascularisation, stimulation of fibroblasts and synthesis of collagen (Rao et al 1996). There are three stages of wound healing, i.e. inflammation, proliferation, and remodelling. The proliferative phase of wound healing demonstrates angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction (Naguib et al 2004). Since both methanolic and aqueous extract increases the number of fibroblasts, collagen tissue and causes complete epithelialization, they may have a significant effect on the proliferative phase of wound healing.

Tannins, triterpenoids and flavonoids are known to promote the wound-healing process. Triterpenoid and flavonoids accelerate wound healing due to their astringent and antimicrobial properties (Singh et al 2014). This seems to be responsible for wound contraction and increased rate of epithelialisation (Ya et al 1988; Tsuchiya et al 1996; Scortichini et al 1991). There are reports of presence of saponins, tannins & terpenoids in Barringtonia acutangula fruits (Khatib and Patil 2011). Fruit of Barringtonia acutangula showed the presence of triterpenoid sapogenins (Barua et al 1968). So the wound healing activity of methanolic and aqueous extract may be attributed to presence of terpenoids.

Staphylococcal infections are among the most common Gram +ve bacterial infections. The wound infections are one of the common staphylococcal infections. Similarly Escherichia coli is a facultatively anaerobic, motile, Gram-negative bacillus whose natural habitat is the large intestine, wherein most strains are commensals, but it can cause serious infection if introduced into the body at sites other than the alimentary tract. These two bacteria were used to infect the wounds. The percentage of wound contraction significantly decreases when the extract were applied on infected wound. This is in agreement with other studies. (Lodhia et al 2009; Ananthanarayan et al 2010) The presence of systemic infection may have decreased the immunity because of which there was such a delay in wound healing (Reddy et al 2008)

The tensile strength of wound represents the effectiveness of wound healing. Usually, wound healing agents promote the gaining of tensile strength. Tensile strength
is the force required to open the healing skin. It is used to measure the completeness of the healing (Lee et al 1968). Both methanolic and aqueous extract showed dose dependent increase in tensile strength (g/cm²) in both normal and infected wound models (Mythreyi et al 2008)

The ethanolic fruit extract shows anti-inflammatory property by augmenting antioxidant defence system in the inflammation bearing rat (Muralidhar et al 2013). Ethanol extract also inhibits prostaglandin synthesis (Sandhyarani et al 2014). There are reports of antioxidant and free radical scavenging activity of various parts of the plant (Babre et al 2010; Kathirvel et al 2012, Sandhyarani et al 2014, Asaduzzaman et al 2015). So the anti-inflammatory and antioxidant activity of fruits of the plant may further contribute to its wound healing activity.

Methanolic extract was more efficacious than aqueous extract in normal rats. It showed > 90% wound contraction on 12th wounding day. However, both methanolic and aqueous extracts showed similar wound healing in infected wounds. So, methanolic extract was chosen for further study. The methanolic extract was evaluated for antibacterial activity and wound healing activity in diabetic rats. Streptozotocin was used to induce diabetes in rats.

Microorganisms are the major cause of many diseases. Now a day, they are resistance to the newly available synthetic drugs (Frieden 2013). So, to combat against the multidrug resistance bacteria, herbal source is the best way. As herbal agents are easily available, cost effective and have less side effect, 80% world population relay on it (Verma et al 2008).

The antibacterial activity of methanolic fruit extract was evaluated by nutrient agar disc diffusion method. Both Gram positive bacterial strains of E. faecalis, S. aureus, A. bumannii and Gram negative bacterial strains C. freundii, E. Aerogenes, E. Coli were used. The zone of inhibition was measured, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) was calculated. The methanolic fruit extract of Barringtonia acutangula showed antibacterial activity against gram positive and gram negative bacteria. So it may possess broad spectrum activity. However the extract showed more significant activity against gram positive bacteria. The less activity against gram negative bacteria may be due to the presence of an outer
membrane that possesses hydrophilic polysaccharides chains as a barrier for extract (Inovye et al 2001). Out of these selected organisms, *S. aureus* causes a variety of suppurative infections in human. It causes superficial skin lesions; more serious infections such as pneumonia, meningitis, urinary tract infections, food poisoning (Lodhia et al 2009), whereas *E. coli* can cause serious infection if introduced into the body at the site other than the elementary tract (Ananthanarayan 2010). The interesting antimicrobial activity against the tested microorganisms could be due to the presence of flavonoids, naphtoquinone and triterpene as reported previously (Ozcelik et al 2008; Chen et al 2015; Ferreira et al 2014). Tannins are known antimicrobial agents that could inhibit the growth of microorganisms by precipitating the microbial protein and thus depriving them of nutritional proteins needed for their growth and development (Obasi et al 2010).

Streptozotocin is a glucosamine-nitrosourea compound. It is an alkylating agent. It is toxic to cells by causing damage to the DNA (Szkudelski et al 2001). Streptozotocin is similar enough to glucose to be transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to beta cells, since these cells have relatively high levels of GLUT2 (Wang et al 1998; Schnedl et al 1994). There are several studies on wound healing activity (Shetty et al 2013, Khatib and Patil 2011, Gregory et al 2014) in streptozotocin (45 mg/kg i.p.) induced diabetic rats. So we used this model to induce diabetes.

The development of wounds is a serious complication for patients with diabetes. Numerous factors related to diabetes can impair wound healing, including wound hypoxia (inadequate oxygen delivered to the wound) infection, nutrition deficiencies, and the disease itself (Lavery et al 2007). Fluctuating blood sugar and hypoxia from poor circulation may impair the ability of white blood cells to destroy pathogenic bacteria and fungi, increasing infection risk (Stadelmann et al 1998). Since The methanolic fruit extract of *Barringtonia acutangula* showed antibacterial activity against both gram positive and gram negative bacteria, its wound healing efficacy in diabetic rats may be attributed in some part to its antibacterial activity.

Diabetic rats show delayed epithelialization period compared to non-diabetic rats (Mekala et al 2014). Wound healing occurs as a cellular response to injury and involves
activation of fibroblast, endothelial cells and macrophages. In diabetic ulcers, healing impairment is caused by a number of physiological factors including diminished fibroblast proliferation decreased angiogenesis and persistent inflammatory response (Galiano et al 2004; Naguib et al 2004). Therefore, agents that stimulate fibroblast growth, promote angiogenesis and relieve inflammation may improve diabetic wound healing (Shetty et al 2013). In our study, MEBA significantly decreased the epithelialization period. The histopathological study reveals that MEBA 20% w/w ointment increases number of fibroblasts, collagen tissue and causes complete epithelialization. So, MEBA may have significant effect on the proliferative phase of wound healing in diabetic rats.

Hydroxyproline, an amino acid, is the major component of collagen which provides strength and support to the tissues. The liberation of hydroxyproline may be due to collagen breakdown. Hence its measurement can be used as an index for collagen turnover as well as a biochemical marker for tissue collagen (Nayak et al 2009). Methanolic extract of Barringtonia acutangula (MEBA) significantly increased the hydroxyproline content thereby influencing the collagen turnover. In addition methanolic extract of Barringtonia acutangula (MEBA) fruits increase tensile strength in dose dependent manner. This shows its effectiveness in wound healing of diabetic rats. There are reports of positive effects of combination of transforming growth factor and fibroblast growth factor on biochemical parameters of wound healing and the tensile strength deficit of diabetic wounds. An increase in tensile strength and hydroxyproline content of treated wounds in the present study may be due to increase in collagen concentration and stabilization of fibres (Ramachandra et al 2012).

The more precise information in qualitative analysis of plant extract can be obtained by GC-MS. This technique also plays an important role for quantitative determination of bioactive phytoconstituents (Serkakkanim et al 2012). Literature survey revealed that, no extensive research work has been reported on GC-MS analysis of methanol extract of Barringtonia acutangula fruits. In our study, methanol extract of fruit of this plant showed the presence of a total of ten bioactive compounds.

A conjugated saponin, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one, is reported to possess strong antioxidant, anticancer, antiproliferative, and anti-
inflammatory properties (Sharma et al 2014.) More recently, anti-inflammatory and cytotoxicity activities of hexadecanoic acid, methyl ester have been reported by (Othman et al. 2015). Glycerin decreases intracranial pressure in numerous disease states, including Reye’s syndrome, stroke, encephalitis, meningitis, pseudotumor cerebri, central nervous system tumor, and space occupying lesions. It is also effective in lowering intraocular pressure in glaucoma and shrinking the brain during neurosurgical procedures. (Ghosh et al 2015) The compound, n-Hexadecanoic acid was known to have anti-inflammatory, antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, flavour, hemolytic, 5-alpha reductase inhibitor. (Vasudevan et al 2012). An antioxidant compound, n-Hexadecanoic acid also possesses wound healing activity by contracting and elevated rate of epithelialisation (Subalakshmi et al 2014). Linoleic acid, 9,12-Octadecadienoic acid (Z,Z)- possesses anti-inflammatory, nematicide, insectifuge, hypocholesterolemic, cancer preventive, hepatoprotective, antihistaminic, antiacne, antiarthritic and antieczemic activities (Sermakkani et al 2012). Octadecanoic acid, a stearic acid identified in methanolic extract was found to have cancer preventive and insectifuge activity. (Ananthi et al, 2013). Similarly, Octadecanoic acid, methyl ester, 6-Octadecenoic acid, (Z)-and 8,11-Octadecadienoic acid were reported as urine acidifier, arachidonic acid-Inhibitor, Inhibitor of production of uric acid and urinary-acidulant. (Dr. Duke's Phytochemical and Ethnobotanical Databases). Cancer preventive, insectifuge, antioxidant, hypcholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, haemolytic activities were also shown by 8-Octadecenoic acid, methyl ester. (Vijisaral et al 2014). Therefore, we assume that the strong bioactivities exhibited by Barringtonia acutangula fruit in this study are correlated to the occurrence of these bioactive compounds in the methanolic extract.

Out of these ten identified compounds, eight compounds were found to have antimicrobial activity. The four major antimicrobial compounds include Glycerin (32.25%) (Triveni et al 2015), 9, 12-Octadecadienoic acid (Z, Z)- (15.63%) (Wei et al 2011), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (14.22%) (Gopa et al 2013), and n-hexadecenoic acid (12.52%) (Kalaivani et al 2012). The other four minor compounds present in the methanol extract of the fruit as Octadecanoic acid (3.33%) (Prabhadevi et al 2012), 8-octadecenoic acid methyl ester (3.11%), Octadecanoic acid,
methyl ester (0.66%) (Meechaona et al 2007), and Hexadecanoic acid, methyl ester (2.44%) were reported to have antibacterial activity (Wei et al 2011).

The antimicrobial activity of methanol extract of *Barringtonia acutangula* fruit may be attributed due to presence of bioactive compounds such as Glycerin, 9,12-Octadecadienoic acid (Z,Z)-, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, n-hexadecenoic acid, Octadecanoic acid, 8-octadecenoic acid methyl ester, Octadecanoic acid, methyl ester and Hexadecanoic acid, methyl ester, which are in agreement with the previous report. Therefore, we assume that the strong antibacterial activity exhibited by *Barringtonia acutangula* fruits in this study is correlated to the occurrence of these bioactive compounds in the methanolic extract.

Structural based virtual screening approach was adopted to identify the compound that is likely to be liable for wound healing activity among the different constituents present in the methanolic extract of *Barringtonia acutangula* fruit. Components that were identified by GC-MS method from methanolic extract of *Barringtonia acutangula* fruit was docked against Glycogen synthase Kinase-3β (GSK-3β, PDB ID 1Q5K). There are evidences 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. Several in silico studies have also been carried out on GSK3-β to study the binding models of different drug molecules (Naika et al 2015, Rao et al 2015).

In GSK-3β, 27 amino acids residue formed the active site pocket of the protein. These were Ile62, Gly63, Val70, Tyr71, Gln72, Ala73, Leu81, Val82, Ala83, Ile84, Lys85, Val110, Leu132, Asp133, Tyr134, Val135, Pro136, Glu137, Thr138, Arg141, Gln185, Asn186, Leu187, Leu188, Leu189, Asp190 and Lys197 (Naika et al, 2015).

Binding mode analysis showed the compound CAS-28564-83-2 (docking score: -6.61) had showed very good binding affinity in comparison to hydroxyproline (docking score: -4.6) and other constituents of *Barringtonia acutangula* fruit extract. The docking pose reveals that both hydroxyproline and CAS-28564-83-2 has similar binding mode with the target protein both forming hydrogen bonding interaction with Leu188 as shown in the figure. The better activity of compound CAS-28564-83-2 might be due to
an extra hydroxyl group present on the compound and also the presence of a double bond in the ring make the compound more rigid and it get better fit into the active site pocket where as hydroxyproline is more flexible. The inactiveness of the other compounds may be due to the lack of hydrogen bond interactions in active site pocket with Leu188.

Hydroxyproline strongly inhibited GSK-3β. Hydroxyproline is 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. As the structure of 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(CAS- 28564-83-2) is very much similar with hydroxyproline there is anticipation that compound may likely to play a major role in healing activity (Naika et al 2015, Rao et al 2015).

Thus, the present study justifies the potent wound healing activity of the phytoconstituents present in Barringtonia acutangula, thereby justifying its traditional use. The wound healing activity of methanolic extract of Barringtonia acutangula in normal, infected and diabetic rats may be attributed to the presence of 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(CAS- 28564-83-2) and n-hexadecenoic acid for their GSK3-β inhibition activity and antibacterial activity.