Healing effect of Dillenia indica fruit extracts standardized to betulinic acid on ultraviolet radiation-induced psoriasis-like wounds in rats

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\begin{abstract}
\textbf{Context:} Dillenia indica Linn. (Dilleniaceae) is traditionally used to treat skin inflammation.

\textbf{Objective:} This study evaluated the healing effect of Dillenia indica fruit extracts on induced psoriasis-like wounds in Wistar rats.

\textbf{Materials and methods:} Extracts were standardized to betulinic acid, including an aqueous ethanolic extract (AEE), ethyl acetate extract (EAE) and petroleum ether extract. Effects against lipid peroxidation were assessed in vitro. Wounds were created at rat tails (n = 12). Topical treatments were applied once daily for 7 days (1 mL of AEE or EAE at 5 or 50 mg/mL). Maximal dose was defined by the extract solubility. A 10-fold lower dose was also tested. Positive and negative controls were treated with clobetasol (0.5 mg/mL) or excipient. Half of each group was euthanized for histology. The remaining animals were observed for 20 days for wound measurements.

\textbf{Results:} Yields of AEE and EAE were 4.3 and 0.7%, respectively. Betulinic acid concentrations in AEE and EAE were 4.6 and 107.6 mg/g. Extracts neutralized lipid peroxidation in vitro at 0.02 µg/mL, accelerating healing at 50 mg/mL. Complete healing in mice treated with AEE occurred 16 days after wound induction. This time was 14 and 12 days in mice treated with EAE and clobetasol. Compared to orthokeratosis, para- keratosis was reduced by AEE (25%), EAE (45%) and clobetasol (55%). EAE caused superior protection against biomolecules oxidation of skin compared to AEE.

\textbf{Discussion and conclusion:} EAE exhibited activity closer to that of clobetasol. Betulinic acid may be an active constituent, which should be assessed in future studies.
\end{abstract}

\section*{Introduction}

\textit{Dillenia indica} Linn. (Dilleniaceae) is an evergreen tree originally from tropical Asia that is well distributed in other countries, such as India, Indonesia, Sri Lanka, Malaysia, Thailand and Vietnam. This plant is known primarily as ‘elephant-apple’ because the fruits are relished by elephants, which are important seed dispersers for this tree (Apu et al. 2010; Gandhi & Mehta 2013). \textit{Dillenia indica} (D. indica) is known as ‘árvore-da-pataca’ or ‘dílenia’ in Brazil, and it was introduced during the nineteenth century (USDA/ARS 1995). The plant grows well in the country’s coastal regions.

Cultural aspects distinguish the natural relationships between plants and people in Asia, where \textit{D. indica} is native, and Brazil, where it was introduced. This tree possesses a large crown and beautiful white aromatic flowers, and it has been used in Brazil primarily for ornamental purposes (Lorenzi et al. 2003). The entire plant is used for medicinal purposes in Asia, and the fruits are used in the cuisine (Gandhi & Mehta 2013).

Asians use the aerial parts of \textit{D. indica} in oral or topical preparations to treat abdominal and joint pain, cough, diarrhoea, fever, tumours, diabetes, toning up the nervous system and removing fatigue (Shome et al. 1980; Sharma et al. 2001; Sood et al. 2005; Yeshwante et al. 2009). Most traditional uses of \textit{D. indica} in folk medicine are associated with anti-inflammatory purposes (Yeshwante et al. 2009). Popular allegations in Brazil corroborate this use, particularly the fruits, which are used in preparations for skin applications to treat inflammation (Lorenzi et al. 2003; Franco 2012).

\textit{Dillenia indica} was investigated scientifically, and these studies demonstrated numerous pharmacological activities, such as anti-inflammatory, antimicrobial, antidiabetic, hypolipidemic and anti-diarrhoeal effects of extracts from leaves (Yeshwante et al. 2009; Apu et al. 2010; Kumar et al. 2011; Rahman et al. 2011; Khare et al. 2013). Antinoceptive and antioxidant activities were demonstrated for the methanolic extract of \textit{D. indica} bark (Alam et al. 2012), and anti-leucemic, anti-diarrhoeal and anti-inflammatory actions were demonstrated for extracts from the fruit of \textit{D. indica} (Kumar et al. 2010; Migliato et al. 2011; Rahman et al. 2011).

The literature reveals that different parts of \textit{D. indica} contain many primary and secondary metabolites. The plant is a rich...
source of triterpenoids, flavonoids, tannins and other less abundant constituents (Gandhi & Mehta 2013). The rich content of flavonoids and triterpenoids in a fraction from leaves exhibited anti-inflammatory activity (Khare et al. 2013). However, most of these previous studies did not evaluate standardized extracts or isolated substances, which makes it difficult to infer the role of any particular plant constituent.

Kumar et al. (2010) standardized the methanol extract from the fruits of D. indica to betulinic acid, which is a natural lupane-type pentacyclic triterpenoid. This acid was considered one of the major bioactive compounds in a study that reported betulinic acid on ultraviolet (UV) radiation-induced psoriasis-like wounds in rats. The Perry betulinic acid on ultraviolet (UV) radiation-induced psoriasis-

Materials and methods

Plant material

Fruits of D. indica were harvested in the gardens of the Universidade do Sul de Santa Catarina (Unisul) in Tubarão, Santa Catarina, Brazil (July 2013). Professor Jasper José Zanco from the same University evaluated plant authenticity. A voucher specimen was deposited at Herbarium Laelía purpurea in Unisul (SRS5103). This study respected all rules of biological biodiversity.

Extraction

Fresh ripe fruits were milled and maintained in shake-assisted macerators in a solvent at 1:2 (w/v) for 2 days, which was repeated three times. Three solvents were used to produce an aqueous ethanolic extract (AEE) made with water:ethanol (1:8), an ethyl acetate extract (EAE) made with ethyl acetate alone (≥99%), and a petroleum ether extract (PEE). All solvents were Vetec® ACS grade reagents. Values of dielectric constants (ε) assumed for ethyl acetate (6.02), petroleum ether (4.03), water (78.36) and ethanol (24.30) were based on the literature. The dielectric constant of the mixture water:ethanol (1:8) was calculated using the ratio ε = ε₂ε₀f₂ + ε₁ε₀f₁, with ε₂ and ε₁ representing the dielectric constants of water and ethanol, respectively, and f₂ and f₁ their volume fraction, respectively (Lund 2009). Solvents were eliminated under reduced pressure after extractions. Extraction performance was calculated in terms of dried extract yield (%), based on the mass of the starting material (Handa 2008).

Quantification of betulinic acid

Betulinic acid in the extracts was quantified using high-performance liquid chromatography (HPLC) in a Shimadzu chromatography workstation (Shimadzu, Japan) assembled with a pump Shimadzu LC10AD, CLASS-agent data management software, a Shimadzu SPD-10A HPLC-detector (UV/VIS) and a Shimadzu CTO10AS VP oven. The protocol was based on previously described methods (Oliveira et al. 2002; Kumar et al. 2010). HPLC grade solvents were obtained from Vetec Química Fina Ltda. Precisely weighed samples of extracts were dissolved in methanol (AEE at 5 mg/mL and EAE at 10 mg/mL) in an ultrasonic bath (Thornton®, Brazil) and passed through a 0.45 μm filter (Millipore, Billerica, MA). Aliquots of 20 μL were injected onto a C₁₈ column (4.6 × 150 mm, 5 μm particle size, Phenomenex Luna®, Torrance, CA). The mobile phase was acetonitrile-phosphoric acid 0.25% (9:1), pH 3.0, which was pumped at a constant flow rate of 1 mL/min (isocratic elution). Eluates were monitored at 210 nm. This analytical method was validated previously. A calibration curve (not shown) was constructed using standard solutions of betulinic acid (Sigma-Aldrich Cat. 91466, ≥97.0). Linear regression analysis data for the validation plots revealed a good linear relationship, with an r² = 0.9994 in the concentration range of 25–300 μg/mL with respect to the peak area. The repeatability (1.2%) of this analytical method was performed using six replicates of the same sample, and the results are expressed in percentages relative to the standard deviation. The detection and quantification limits were 0.075 and 0.25 μg/mL, respectively. Recovery percentages from AEE and EAE were 97.3 ± 3.4% and 98.8 ± 2.9%, respectively. Therefore, the method was considered acceptable under the stated operational conditions. Data are expressed in mg of betulinic acid per gram of extract.

Lipid peroxidation test in vitro

Egg yolk was used as a source of lipids (Petronilho et al. 2012; Müller et al. 2016). Yolks were homogenized (1% w/v) in 20 mM phosphate buffer (pH 7.4). The free radical-generating compound 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH, Aldrich Cat. 440914) was used at 120 μM (1.9 v/v) to induce lipid peroxidation. Extracts were added to the reaction mixture at 0.02 to 2 μg/mL. Clobetasil propionate (Sigma-Aldrich Cat. C8037) was used at 100 μM (46.7 μg/mL) as the positive control (Iaques et al. 2012). Reactions were performed for 30 min at 37°C. Aliquots of each sample (0.5 mL) were centrifuged with 0.5 mL of 15% trichloroacetic acid (Sigma-Aldrich Cat. T6399, Torrance, CA) at 1200 g for 10 min. The supernatants (0.5 mL) were mixed with 0.5 mL of 0.67% thiobarbituric acid (Sigma-Aldrich Cat. T5500) and heated at 95°C for 30 min (Scola et al. 2011). This reaction generated thiobarbituric acid reactive substances (TBARS), which are formed as by-products of lipid peroxidation. Malondialdehyde (MDA) is one of several end products formed via the decomposition of certain lipid peroxidation products. The absorbance was measured at 532 nm (Scola et al. 2011). Data are expressed as concentrations (nmol) of MDA normalized by the protein content (Lowry et al. 1951).

Animals

The Animal Care and Use Committee from the Universidade do Sul de Santa Catarina approved this protocol (13.032.4.03.IV).
All animal experiments were performed in accordance with the National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978). Male albino Wistar rats (250 ± 20 g) were housed under controlled conditions (12 h light/dark cycle, 22 ± 2°C, 60% air humidity) and had free access to food and water. Animals were acclimatized for at least 5 days before experiments.

**Wound induction and treatment**

The upper medial part of the rat tail (5 cm) was irradiated for 30 min (1.5 J/cm²) at a vertical distance of 20 cm with UV lamps (Philips, Brazil) to induce skin wounds (day zero) (Vogel & Vogel 2002). Rats were divided into six groups (n = 12): a negative control treated with excipient (1 mL of ethanol:water 1:2) and a positive control treated with clobetasol propionate (Sigma-Aldrich Cat. C8037) at 0.5 mg/mL (1 mL). Clobetasol is a corticosteroid anti-inflammatory drug that is used to treat psoriasis (Gordon 1998). Four test groups were treated with AEE or EAE at 5 mg/mL or 50 mg/mL (1 mL), respectively. The maximal dose was defined by the solubility of the extracts in the excipient. Extracts were difficult to dissolve in formulations containing >50 mg/mL. A 10-fold lower dose of each extract was also tested. The excipient was chosen based on the macerations of *D. indica* fruits that are generally used traditionally and compatibility with clobetasol (Bruze et al. 1995). Treatments were applied topically to the wound area. No signs of toxicity, such as death, hair erection, dullness, inactivity or loss of appetite, were observed. Treatments were initiated 72 h after wound induction, when wounds were apparent, and repeated every 24 h for 7 days.

**Wound-healing assessment**

Wound healing was monitored using macroscopic and microscopic evaluations. Half of each group (n = 6) was euthanized 24 h after the last dose, and tails were amputated for histology and measurements of biomarkers of oxidative damage in the biomolecules of the skin. The remaining animals were kept alive (n = 6/each group) for daily observation for 20 days to quantify the time required for healing. Pictures were taken using a digital camera (Kodak Easy Share CX7430, Rochester, NY). Images were analyzed using CHPTool 5.0 software (Cyclops Group, Brazil), as previously described (Martins et al. 2011). Wound healing was

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**Table 1. Features of the extraction procedures and data of standardization of extracts to betulinic acid.**

| Solvent            | Extract | Dielectric constant | Yield in dried extract (%) | Betulinic acid (mg/g) | Total betulinic acid extracted (mg)* |
|--------------------|---------|--------------------|---------------------------|-----------------------|-------------------------------------|
| Water:ethanol (1:8) | AEE     | 30.78              | 4.30 ± 0.30               | 4.60 ± 2.50           | 98.90 ± 7.00                        |
| Ethyl acetate      | EAE     | 6.02               | 0.70 ± 0.03               | 107.60 ± 5.00         | 360.50 ± 12.00                      |
| Petroleum ether    | PEE     | 4.03               | 0.06 ± 0.01               | 0                     | 0                                   |

*Extracted from starting plant material 500 g.
determined using the following formula:

\[
\text{Wound healing} \% = \left( \frac{\text{Healed area}}{100} \right) / \text{Wounded area}
\]

**Histology**

Cutaneous tissues were fixed in formaldehyde 10% (24 h). Tissue was embedded in paraffin, sliced vertically using a microtome (Leica Biosystems RM2235, Germany) and stained with haematoxylin (Merck Cat. 115938) and eosin (Merck Cat. 115935) (Yuan et al. 2010). Slides were examined under a Olympus CX41 microscope, and a Olympus Image Analysis Software (Olympus, Japan) was used to quantify the following factors: (a) Parakeratosis, which is a keratinization that is characterized by an aberrant retention of nuclei in the stratum corneum; (b) Orthokeratosis, which is the formation of a nuclear keratin layer in the normal epidermis and (c) quantification of infiltrating inflammatory cells. The analysis was performed in technical triplicates with tissue samples of six mice from each group (biological replicates) (Vijayalakshmi et al. 2012). Parakeratosis and orthokeratosis were quantified using an adaptation of the method described previously by Wolberink et al. (2011). The surface percentage of total horizontal involvement of parakeratosis compared to orthokeratosis was first estimated visually and categorized in four groups: zero, <30, between 30 and 70 and >70 up to 100% (Figure 1(C–F), respectively). The analysis was carried out always by the same observer. Then, a score value was given respectively to each category in the 1–4 range (1 – absent, 2 – minimum, 3 – moderate and 4 – intense), resulting in a mean score for each group. The results were expressed in percentages compared to the negative control, being the value of score 4 the representation of maximal predominance of parakeratosis over orthokeratosis.

**Biomarkers of oxidative damage in biomolecules of tail skin**

Lipid peroxidation was measured using the TBARS reaction (Ohkawa et al. 1979). Homogenates of fresh skin tissue from the rat tails were prepared in buffer (30 μM Na₂PO₄ and 140 μM KCl, pH 7.4) at 1:2 (w/v). Samples were centrifuged (10 min, 1000 g), and supernatants were mixed with 10% trichloroacetic acid (1:3) (Sigma-Aldrich Cat. T6399), and centrifuged. Thiobarbituric acid (0.67 %, 1:2 v/v) (Sigma-Aldrich Cat. T5500) was added, and the solution was heated (95°C, 30 min). Absorbances were read at 532 nm. Carbonyl proteins were measured in homogenates prepared in buffer (40 μM KH₂PO₄ and 120 μM KCl pH 7.4). Carbonyl groups in the sample react covalently with 2,4-dinitrophenylhydrazine (Aldrich, Cat. D199303) to form a 2,4-dinitrophenylhydrazone, which is quantifiable at 370 nm (Levine et al. 1990). These assays were performed in technical triplicates with tissue samples of six mice from each group (biological replicates). All data were normalized by protein content (Lowry et al. 1951).

**Statistical analysis**

Data are expressed as the means ± standard deviation or percentages. The assays were performed in technical triplicates. Data were analyzed using analysis of variance (ANOVA) and the Bonferroni test. Comparisons and differences were processed using GraphPad Prism software (San Diego, CA). Values of \( p < 0.05 \) were considered statistically significant.
acid of analytical standard grade and the major peak corresponding to betulinic acid, which appeared at a retention time of 4.6 min. Figure 2(B,C) show the chromatograms of AEE and EAE, respectively. Both chromatograms exhibited peaks that corresponded to betulinic acid. Table 1 shows the calculated concentrations of betulinic acid in AEE and EAE, and the total of betulinic acid extracted from the starting plant material (500 g). The concentration of betulinic acid in EAE was >20-fold higher than AEE (Table 1).

Figure 2(D) shows the phytochemical screening data of extracts. The main difference between AEE to EAE was the content of steroids and triterpenes. This result is consistent with the HPLC data and confirmed that the extraction performed using ethyl acetate was more efficient at extracting triterpenoid compounds, such as betulinic acid. Figure 2(D) shows that considerable amounts of flavonoids were also identified in AEE and EAE.

Ethanol and ethyl acetate are good solvents for betulinic acid (Cheng et al. 2011). The presence of water in the extraction of betulinic acid using ethanol possibly reduced the yield because water increases the dielectric constant of the solvent. Betulinic acid using ethanol possibly reduced the yield because water increases the dielectric constant of the solvent. Betulinic acid of analytical standard grade and the major peak corresponding to betulinic acid, which appeared at a retention time of 4.6 min. Figure 2(B,C) show the chromatograms of AEE and EAE, respectively. Both chromatograms exhibited peaks that corresponded to betulinic acid. Table 1 shows the calculated concentrations of betulinic acid in AEE and EAE, and the total of betulinic acid extracted from the starting plant material (500 g). The concentration of betulinic acid in EAE was >20-fold higher than AEE (Table 1).

Kumar et al. (2010) registered a similar concentration (97 mg/g) compared to the EAE data obtained in the current work (107.6 mg/g). The current work used a one-step procedure, and Kumar et al. (2010) used methanolic maceration followed by liquid–liquid fractionation.

The increased generation of reactive species leads to the molecular damages observed in inflammatory processes, such as in psoriasis (Kadam et al. 2010). Some in vivo studies in rats demonstrated that betulinic acid attenuated oxidative stress in experimental inflammation models (Lingaraju et al. 2015). Therefore, the promising wound-healing properties of AEE and/or EAE may be associated with some induced protection against oxidative damage. The extracts were screened initially for protection against in vitro lipid peroxidation induced by AAPH from lipids in egg yolk (Figure 3). AEE and EAE provided significant protection against lipid peroxidation and neutralized AAPH at concentrations as low as 0.02 μg/mL (p < 0.001). EAE exhibited subtly more protection at 2 μg/mL.

Figures 1, 4–5 show the in vivo effects on radiation-induced rat tail wounds. Figure 4(A–F) shows bar diagrams of wound healing over time using macroscopic examinations. Closed bars correspond to wounds during the repair process, and open bars correspond to completely healed wounds. Data of the negative controls (Figure 4(A)) show that wound healing was prolonged in this group. Wound healing in the negative control was nearly 40% on day 10, and not all animals achieved complete healing on day 20. Figure 4(C,D) shows that AEE or EAE treatments at 5 mg/mL did not accelerate wound healing. However, treatments with clobetasol at 0.5 mg/mL (Figure 4(B)), AEE at 50 mg/mL (Figure 4(E)) or EAE at 50 mg/mL (Figure 4(F)) induced significant acceleration of wound healing. EAE was the extract that exhibited the most promising wound-healing activity (Figure 4(F)). The activity of EAE was closer to clobetasol. EAE at 50 mg/mL produced wound healing (nearly 60%) on day 8, and wounded tissues had repaired completely on day 14 (Figure 4(F)). Figure 4(G) illustrates the evolution of wound healing in rats treated with EAE (50 mg/mL). Wound healing in rats treated with AEE at 50 mg/mL was nearly 60% on day 10, and apparent complete repair was registered on day 16 (Figure 4(E)). Only samples of skin tissue from animals treated with 50 mg/mL extracts were subjected to the following evaluations because of the ineffectiveness of AEE and EAE at 5 mg/mL.

Figure 1 shows the microscopic evaluation data (histology). The Perry’s scientific tail model uses UV radiation to induce skin wounds and inflammation. This model cannot reproduce all of the dysfunctions of keratinocytes and the immune-mediated processes observed in psoriasis, but it is accepted as a screening method for assessments of anti-psoriatic activity because it increases parakeratosis and reduces orthokeratosis. An induction of orthokeratosis in areas of scale of epidermis (between the follicles) can be taken as an indicator of anti-psoriatic activity (Hofbauer et al. 1988; Vijayalakshmi et al. 2012). The microscopic findings revealed that EAE at 50 mg/mL restrained parakeratosis compared to orthokeratosis in up to 45% (Figure 1(A)), whereas clobetasol did in nearly 35% compared to the negative control (p < 0.001). AEE restrained parakeratosis compared to orthokeratosis in nearly 25% (Figure 1(A)). EAE produced superior reductions in immune cell infiltration in granulation tissue compared to AEE (p < 0.05), and these results were closer to the effects of clobetasol (Figure 1(B)). Figure 1(C) shows an image of a slice of tissue from a positive control showing no parakeratosis and, predominant orthokeratosis instead (score 1). Figure 1(F) shows an image of a tissue slice of a negative control with
intense parakeratosis compared to orthokeratosis (score 4) and
swollen epidermal cones with inflammatory infiltrate.

Figure 5 shows the biomarkers of oxidative damage in biomo-
lecules of tail skin from rats subjected to the experimental treat-
ments. These evaluations were performed to verify whether the
wound-healing effect was associated with a potential protection
of the extracts against oxidative damage in vivo. Two biomarkers,
which normally increase during inflammation, were assessed:
lipid peroxidation, which was a marker of damage on membrane
lipids (Zhao et al. 2014), and the concentration of carbonyl pro-
teins, which was a marker related to protection against protein
modifications. Figure 5(A,B) shows that EAE induced sig-
ificantly superior protection ($p < 0.05$), closer to clobetasol. Tissue
from mice treated with EAE exhibited reduced lipid peroxidation
(up to 60%) and protein carbonylation (up to 40%) compared to
the negative control ($p < 0.001$).
Conclusions

This study supports the traditional claims of the anti-inflammatory activity of *D. indica*. The results suggested that fruit extracts accelerated the healing of psoriasis-like wounds and reduced inflammation via a mechanism associated with protection against oxidative damage in biomolecules. EAE exhibited the most promising activity, and this extract contained the highest concentration of betulinic acid. These results suggest that betulinic acid may be an active constituent, and further studies should evaluate the activity of betulinic acid for the treatment of psoriasis.

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Disclosure statement

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References

Alam MB, Rahman MS, Hasan M, Khan MM, Nahar K, Sultana S. 2012. Antinociceptive and antioxidant activities of the *Dillenia indica* bark. Int J Pharmacol. 8:243–251.

Apu A, Muhit M, Tareq S, Pathan A, Jamaluddin A, Ahmed M. 2010. Antimicrobial activity and brine shrimp lethality bioassay of the leaves extract of *Dillenia indica* Linn. J Young Pharm. 2:50–53.

Bruze M, Isaksson M, Dooms-Goossens A. 1995. The influence of patch tests with clobetasol propionate on adjacent patch test reactions. Contact Dermatitis. 32:167–170.

Cheng Y, Shao Y, Yan W. 2011. Solubilities of betulinic acid in thirteen organic solvents at different temperatures. J Chem Eng Data. 56:4587–4591.

Christensen BV, Abdel-Latif IA. 1949. Colorimetric and fluorometric studies on the Borntraeger reaction for anthraquinone drugs. J Am Pharm Assoc. 38:487–489.

Franco IJ. 2012. Minhas 500 plantas medicinais. Aparecida, Brasil: Editora Santuário.

Gandhi D, Mehta P. 2013. *Dillenia indica* Linn and *Dillenia pentagyna* Roxb: pharmacognostic, phytochemical and therapeutic aspects. J App Pharm Sci. 3:134–142.

Gordon ML. 1998. The role of clobetasol propionate emollient 0.05% in the treatment of patients with dry, scaly, corticosteroid-responsive dermatoses. Clin Ther. 20:26–39.

Handa SS. 2008. An overview of extraction techniques for medicinal and aromatic plants. In: Handa SS, Khanuja SPS, Longo G, Rakesh DD, editors. Extraction technologies for medicinal and aromatic plants. Trieste, Italy: International Centre for Science and High technology; p. 21–52.

Hofbauer M, Dowd PM, Atkinson J, Whitefield M, Greaves MW. 1988. Evaluation of a therapeutic concentration of dithranol in the mouse-tail test. Br J Dermatol. 118:85–89.

Jaques JA, Rezer JF, Ruchel JB, Souza VC, Pinheiro KV, Schlemmer KB, Schlemmer JB, Bertoldo TM, Martins NM, Bertoncelhi CM, et al. 2012. An experimental model of contact dermatitis: evaluation of the oxidative profile of Wistar rats treated with free and nanoencapsulated clobetasol. Redox Rep. 17:206–213.

Jingbo W, Aimin C, Qi W, Xin L, Huaining L. 2015. Betulinic acid inhibits IL-1β-induced inflammation by activating PPAR-γ in human osteoarthritis chondrocytes. Int Immunopharmacol. 29:687–692.

Kadam DP, Suryakar AN, Ankush RD, Kadam CY, Deshpande KH. 2010. Role of oxidative stress in various stages of psoriasis. Indian J Clin Biochem. 25:388–392.

Khare RK, Prasad AK, Kumar S, Iyer SV, Vaidya SK, Bigoniya P. 2013. Flavonoid and triterpene rich fraction of *Dillenia indica* Linn. leaves: as anti-inflammatory & anti-arthritic activity. Indo Am J Pharm Res. 3:4653–4659.
Khoddami A, Wilkes MA, Roberts TH. 2013. Techniques for analysis of plant phenolic compounds. Molecules. 18:2328–2375.

Kumar D, Mallick S, Vedasirromoni JR, Pal BC. 2010. Anti-leukemic activity of Dillenia indica L. fruit extract and quantification of betulinic acid by HPLC. Phytochemistry. 71:431–435.

Kumar S, Kumar V, Prakash O. 2011. Antidiabetic, hypolipidemic and histopathological analysis of Dillenia indica (L.) leaves extract on alloxan induced diabetic rats. Asian Pac J Trop Med. 4:347–352.

Levine RL, Garland D, Oliver CN, Amici A, Lenz AG, Ahn BW, Shaltiel S, Stadtmann ER. 1990. Determination of carbonyl content in oxidatively modified proteins. Meth Enzymol. 186:464–478.

Lingaraju MC, Pathak NN, Kandasamy K, Kumar D, et al. 2015. Betulinic acid attenuates renal oxidative stress and inflammation in experimental model of murine polymicrobial sepsis. Eur J Pharm Sci. 70:12–21.

Lorenzi H, Souza HM, Torres MAV, Bacher LB. 2003. Árvores exóticas no Brasil: madeireiras, ornamentais e aromáticas. Nova Odessa, SP: Instituto Plantarum.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193:265–275.

Lund W. 2009. The pharmaceutical codex: principles and practice of pharmaceutical pain and glial activation in the spinal cord and enhances nerve regeneration in rats. Pain. 152:2633–2661.

Migliato KF, Chiosini MA, Mendonca FA, Esquisatto MA, Salgado HR, Santos GM. 2011. Effect of glycolic extract of Dillenia indica L. combined with microrcurrent stimulation on experimental lesions in Wistar rats. Wounds. 23:111–120.

Müller SD, Florentino D, Ortmann CF, Martins FA, Danielski LG, Michels M, de Souza Constantino L, Petronilho F, Reginato FH. 2016. Anti-inflammatory and antioxidant activities of aqueous extract of Cecropia glaziovii leaves. J Ethnopharmacol. 185:255–262.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thioarbituric acid reaction. Anal Biochem. 95:351–358.

Oliveira BHD, Cid AM, Santos CAM, Espindola APDM. 2002. Determination of the triterpenoid, betulinic acid, in Dolichocarpus schottianus by HPLC. Phytochem Anal. 13:95–98.

Peach K, Tracey MV. 1956. Modern methods of plant analysis. Berlin, Germany: Springer Verlag.

Petronilho F, Dal-Pizzol F, Costa GM, Kappel VD, de Oliveira SQ, Fortunato J, Cittadini-Zanette V, Moreira JC, Simões CM, Dal-Pizzol F, et al. 2012. Hepatoprotective effects and HSV-1 activity of the hydroethanolic extract of Ceropegia glaziovii (embauta-vermelha) against acyclovir-resistant strain. Pharmacol Biol. 50:911–918.

Rahman MS, Shams-UD-Doha KM, Roksana R. 2011. Antidiarrhoeal activity of the leaf and fruit extracts of Dillenia indica. Int J Biosci. 1:39–46.

Scola G, Kappel VD, Moreira JCF, Dal-Pizzol F, Salvador M. 2011. Antioxidant and anti-inflammatory activities of winery wastes seeds of Vitis labrusca. Cienc Rural. 41:1233–1238.

Sharma HK, Chhantle I, Dolui AK. 2001. Traditional medicinal plants in Mizoram, India. Fitoterapia. 72:146–161.

Shome U, Khanna RK, Sharma HP. 1980. Pharmacognostic studies of Dillenia indica Linn. II – Fruit and seed. Proc Indian Acad Sci. 89:91–104.

Shrivastav S, Sindhu RK, Kumar S, Kumar P. 2009. Anti-psoriatic and phytochemical evaluation of Thespesia populnea barks extract. Int J Pharm Pharm Sci. 1(Supp.1):176–185.

Sood SK, Bhardwaj R, Lakanpal TN. 2005. Ethnic Indian plants in cure of diabetes. Jodhpur, India: Scientific Publishers.

United States Department of Agriculture – Agricultural Research Service (USDA/ARS). 1995. Taxon: Dillenia indica L. [Internet]. US National Plant Germplasm System; [cited 2015 Dec 28]. Available from: https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?id=14122.

Vijayalakshmi A, Ravichandiran V, Malarkodi V, Nirmala S, Jayakumari S. 2012. Screening of flavonoid quercetin from the rhizome of Smilax glauca Linn. for anti-psoriatic activity. Asian Pac J Trop Biomed. 2:269–275.

Vogel GH, Vogel WH. 2002. Drug discovery and evaluation: Pharmacological assays. Berlin Heidelberg, Germany: Springer-Verlag.

Wolberink EAW, van Erp PEJ, Teussink MM, vand de Kerkhof PCM, Gerritsen MJP, van der Kerkhof PCM, Gerritsen MJ. 2011. Cellular features of psoriatic skin: imaging and quantification using in vivo reflectance confocal microscopy. Cytometry B Clin Cytom. 80B:141–149.

Yeshwante SB, Juvekar AR, Nagnotti DM, Wankhede SS, Shah AS, Pinnipikar RB, Saindane DS. 2009. Anti-inflammatory activity of methanolic extracts of Dillenia indica L. leaves. J Young Pharm. 1:63–66.

Yuan B, Wang X, Wang Z, Wei J, Qing C, Lu S. 2010. Comparison of fibro genesis caused by dermal and adipose tissue injury in an experimental model. Wound Repair Regen. 18:202–210.

Zhang Y, Mitiyala S, Miao L, Mitov M, Schnell D, Dhar SK, Cai J, Klein JB, Sultana R, Butterfield DA, et al. 2014. Redox proteomic identification of HNE-bound mitochondrial proteins in cardiac tissues reveals a systemic effect on energy metabolism after doxorubicin treatment. Free Radic Biol Med. 72:55–65.