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The antioxidant capacity and immunomodulatory activity of stingless bee honeys proceeding from Costa Rica

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ABSTRACT

Objective: Medihoney® is considered up to date the only antioxidant therapy specifically approved by the United States food and drug administration for wound healing. This is the first study that evaluates the antioxidant capacity and immunomodulatory activity of stingless bee honeys in comparison to Medihoney®. The goal of this study was to evaluate *in-vitro* the potentials of Meliponini honey from Costa Rica as an antioxidant wound dressing agent. **Materials and Methods:** A total of 57 honeys belonging to the *Tetragonisca angustula* ($n = 36$) and *Melipona beecheii* ($n = 21$) species were studied. Following tests were carried out: Screening of radical scavenging activity by the 2,2-diphenyl-1-picrylhydrazyl assay (DPPH), superoxide anion radical scavenging assay, inhibition of xanthine oxidase (XO), total phenolic (TP) content by the Folin–Ciocalteu reagent, and the oxygen radical absorbance capacity (ORAC) assay. Furthermore, the effect of honey over reactive oxygen species (ROS) produced by human polymorphonuclear leucocytes (PMNL) was evaluated using luminol and a Cypridina luciferin analog as luminescence probes. **Results:** The positive correlation between the TP content and the ORAC value reported herein, suggests that the antioxidant capacities of the stingless bee honeys examined are given in a major grade to their phenolic constituents. According to present results, the *in-vitro* immunomodulatory activities are explained mainly due to the radical scavenging effect and to the XO inhibition. Both mechanisms may play a role in the impairment of the inflammatory process and the promotion of redox homeostasis. **Conclusion:** The *T. angustula* honeys showed antioxidant capacities not statistically different to Medihoney®. In addition, their radical scavenging activity over ROS produced by human PMNLs and inhibition of XO justify their ethnopharmacological use as a wound dressing and further research toward novel wound-healing developments.

KEY WORDS: Honey, immunomodulatory, medihoney, meliponini

INTRODUCTION

The medical applications of *Apis mellifera* (honeybee) honey are ancient. The use of honey as a medicine is present in a myriad of cultures and this practice possesses over four millennia of pharmaceutical history [1]. The advent of the antibiotic era of the 20th century set the medical use of honey aside [2,3]. The subsequent onset of antimicrobial resistance led to the search of alternative treatments for wound healing [4-6], which promoted the resurge of honey as a medicine [7-9]. In recent years, honey-based wound dressings made a breakthrough as validated treatments for wounds and burns. In 2008, supported on its proven clinical efficacy [10], Medihoney® (Derma Sciences) received the food and drug administration (FDA) approval for its application as a wound dressing [11]. Medihoney® is today a treatment for burns and wounds accepted by the medical community and considered up to date the only antioxidant therapy that has been specifically approved for wound healing [12].

The bees (Hymenoptera, Apidae) of the Meliponini tribe are eusocial insects present in the tropical regions of

America, Africa, and Australia. Although taxonomically and morphologically diverse, all share the same feature: The absence of a functional sting; hence known as stingless bees [13]. The ancient Maya and Aztec Mesoamerican cultures were the first to practice the keeping of stingless bees or meliponiculture as is commonly known. They considered the Meliponini bees sacred and used the stingless bee honey in their religious rituals and as a medicine [14,15]. Among the ancient medical applications of the Meliponini honey, its use as a wound dressing and as a treatment for cataracts and conjunctivitis are still common practice in tropical America today [16,17].

The stingless bee honey of Mesoamerica has been mostly subject of study over its antimicrobial activity, physical and chemical quality in comparison to A. *mellifera* honey standards [17,18]. To our knowledge, this is the first study that evaluates the antioxidant capacity and immunomodulatory activity of stingless bee honeys related to their wound healing properties in comparison to Medihoney®. Our goal was to evaluate *in-vitro* the potentials of Meliponini honey from Costa Rica as an antioxidant wound dressing agent.

MATERIALS AND METHODS

Sample Collection

A total of 57 stingless bee honeys took part in the present study. The samples were bought directly from keepers. The hives were located in regions of Costa Rica where meliponiculture is practiced [19]. The same set of samples and reference were the subject of study of a previous investigation by Zamora *et al.* [20]. The honeys under study belonged to the following stingless bee species: *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21). Medihoney® hydrocolloid wound paste (Derma Sciences) was used as a reference for antioxidant capacity.

Reagents and Consumables

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Greiner Bio-One GmbH (Frickenhausen, Germany) provided the microplates. Sanquin bloedbank Noordwest (Amsterdam, The Netherlands) provided the human polymorphonuclear leukocytes (PMNL), which were isolated from blood donated by healthy volunteers. The blood was processed in accordance to local guidelines; every donor signed an informed consent stating that their blood could be used for more than patient care only.

Sample Preparation

An aliquot of 0.9-1 g of the Meliponini honey or Medihoney® was weighed in an analytical balance (Ohaus Corp., Parsippany, NJ, USA). A start solution of 1000 mg/ml of the honey or the reference was prepared for each analysis. The preparation of the solution took on regard the individual density of the sample or the reference in order to achieve comparable results, as reported previously [20]. Every solution was prepared in the appropriate solvent for each test.

Screening of Radical Scavenging Activity by the 2,2-diphenyl-1-picryl hydrazyl (DPPH) Assay

The radical scavenging activity of the stingless bee honeys and Medihoney® over DPPH was executed by means of a microplate method based on the procedure described by Ancerewicz *et al.* [21].

Test solutions of 500 mg/ml of the sample or the reference were prepared in 75% ethanol. Three aliquots of 150 µl were serially diluted with 100 µl of 75% ethanol in a flat-bottomed microplate. Later, 100 µl of a solution of 0.2 mg/ml of DPPH were added to two of the dilution series. 100 µl of 75% ethanol were added to the third series, which was used as blank. The final volume per well was 200 µl. The test included a calibration curve composed of three concentrations of DPPH: 0 (200 µl of 75% ethanol), 50 (150 µl of 75% ethanol and 50 µl of the DPPH solution), and 100% (100 µl of 75% ethanol and 100 µl of the DPPH solution). Once the DPPH solution was added to the serial dilutions and calibration curve, the microplate assay was incubated for 15 min at room temperature (23°C) before measuring absorbance at 550 nm in a Multiskan Spectrum microplate reader with SkanIT DDE software (Thermo Scientific). The scavenging activity was calculated as the concentration (mg/ml) capable of achieving a 50% inhibition of DPPH (IC_{50}). Every sample and the reference were subject of three separate analyses.

Superoxide Anion (O_2^-) Radical Scavenging Assay

The scavenging activity of the test samples over the superoxide anion was determined in a cell-free system as described by Beukelman *et al.* [22]. Every sample and the reference were subject of two separate analyses. The scavenging activity was calculated as the IC_{50}.

Inhibition of Xanthine Oxidase (XO)

The inhibitory effect of honeys on XO was analyzed by means of a kinetic microplate assay. Three aliquots of 100 µl of honey test solutions of a concentration of 200 mg/ml in phosphate buffered saline (PBS, pH 7.4) were serially diluted in an ultraviolet transparent microplate with 50 µl of PBS. Subsequently, 50 µl of hypoxanthine (0.27 mg/ml in PBS) were added to all the dilution series. Later, 50 µl of PBS and finally 50 µl of XO (2.5 mU per well, in PBS) were added. The assay included a control per serial dilution. The control consisted of 100 µl of PBS, 50 µl of hypoxanthine, and 50 µl of XO. The final volume per well was 200 µl. Immediately, uric acid production was monitored by a kinetic measurement of absorbance at 290 nm for 30 min at 23°C (7 absorbance readings, with an interval of 5 min). The average kinetic rate (normal rate) of every well was used for calculating the inhibition of XO as an IC_{50}. The kinetic readings and the average kinetic rate calculations were performed again in the Multiskan Spectrum microplate reader with SkanIT DDE software. Every sample and the reference were subject of three separate analyses.

The effect of honeys over XO was analyzed as a verification of the results obtained in the superoxide radical scavenging assay. Superoxide is generated by means of the reaction of the enzyme XO over hypoxanthine. The goal of this test was to distinguish between superoxide radical-scavenging and inhibition of XO.

Total Phenolic (TP) Content by the Folin–Ciocalteu Reagent

The TP content of the honeys in study was determined by a microplate method based on the publication by Singleton *et al.* [23]. Three aliquots of 320 μl of honey test solutions with a concentration of 20 mg/ml in demineralized water were serially diluted in a microplate with 160 μl of demineralized water. Two dilution series took part of the test reaction and one series was used as blank. For this purpose, 40 μl of demineralized water were added to the blank. The microplate assay included a calibration curve of 11 concentrations of gallic acid (0-12.5 μg/ml). 160 μl of every gallic acid solution were distributed in duplicate. 200 μl of demineralized water were dispensed in two wells as blank of the calibration curve. Subsequently, 10 μl of the Folin–Ciocalteu reagent were added to the test dilution series and the calibration curve. After 5 min, 30 μl of a solution of 200 mg/ml of Na_2CO_3 were added to the dilution series and calibration curve. The final volume per well was 200 μl. Later, the microplate was incubated for 2 h at room temperature (23°C) in a microplate shaker (Thermo Scientific). Finally, the absorbance at 765 nm of the assay was measured in the Multiskan Spectrum microplate reader with SkanIT DDE software. In the present investigation, the TP content values are presented as micrograms of gallic acid equivalents (GAE) per milligram of honey (μg GAE/mg). Every sample and the reference were subject of three separate analyses.

Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC values of the honeys in study were quantified using a microplate assay based on the method described by Huang *et al.* [24]. Two aliquots of 50 μl of a honey test solution with a concentration of 10 mg/ml in 75 mM sodium phosphate buffer (PBS, pH 7.4), were serially diluted in a black microplate with 25 μl of PBS. The microplate assay included a calibration curve of six concentrations (6.25-50 μM) of 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (trolox). 25 μl of every solution of trolox were dispensed in triplicate. Subsequently, 150 μl of an 80 nM fluorescein sodium salt solution were added to the test dilution series and the calibration curve. The test included a blank composed of 50 μl of PBS and 150 μl of the fluorescein solution. Following, the assay was incubated for 10 min at 37°C. Afterward, 25 μl of a solution of 150 mM of 2,2-azobis(2-methylpropionamidine-dihydrochloride) (AAPH) in PBS were dispensed to every well with exception of the blank. The test included a control for the AAPH activity. This control consisted of 25 μl of PBS, 150 μl of the fluorescein solution and 25 μl of the AAPH solution. Right after the addition of the AAPH solution, fluorescence intensity was measured (485 nm [excitation]/525 nm [emission]) at 37°C for 60 min, with an interval between readings of 1-min and under constant stirring in a Fluoroskan FL fluorometer with Ascent software (Thermo Scientific). The ORAC values were calculated as μmol of trolox equivalents per 100 g of honey (μmol TE/100 g). Every sample and the reference were subject of three separate analyses.

Effect Over Reactive Oxygen Species (ROS) Produced by Human PMNLs

The radical scavenging activity of the test samples over human PMNLs stimulated with opsonized zymosan was performed in microplate chemiluminescence assays. 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (Cypridina luciferin analog) were used as luminescence probes. Every sample and the reference were subject of two separate analyses with each probe. This method was described previously [22].

Statistical Analyses

All descriptive statistics, normal distribution evaluation, Pearson correlation tests, one sample sign non-parametric test on median values, and multivariate analysis were performed using MiniTab® software (Minitab Inc., State College, PA, USA).

RESULTS

The test results did not follow a normal distribution. We performed a multivariate analysis for the *M. beecheii* and *T. angustula* honeys taking in regard the results of the following parameters: ORAC value, TP content, the effect over ROS produced by stimulated PMNLs using luminol and Cypridina luciferin analog as luminescence probes, DPPH and XO inhibition [Figure 1].

The principal components analysis (PCA) unveiled that the first component was capable to separate 98.2% of the Meliponini honeys according to species and explains 49.5% of the variance between the data. In addition, both components together explain 74.4% of the variance between the data. High TP content and high ORAC values characterize *T. angustula* samples. Likewise, high IC_{50} values in DPPH and XO inhibition categorize *M. beecheii* honeys.

The Pearson correlation analyses returned three highly significant correlations ($P < 0.001$) between the assays performed to the stingless bee honeys. A positive correlation between DPPH and XO inhibition (Pearson coefficient = 0.695); likewise, a positive correlation between TP and ORAC (Pearson coefficient = 0.848); and finally, a negative correlation between ORAC and XO inhibition (Pearson coefficient = −0,728) was present.

All *T. angustula* samples and Medihoney® possessed efficacy in every test performed. In contrast, 33.3% of *M. beecheii* honeys did not reach an IC_{50} in the DPPH inhibition assay. Figure 2 comprises the results for the antioxidant capacity tests, the immunomodulatory assays and the one sample sign non-parametric tests on median values.

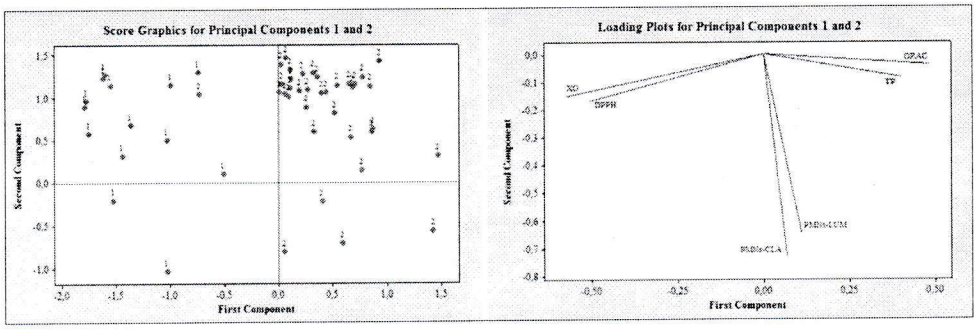

Figure 1: Principal components analysis for stingless bee honeys. 1: *Melipona beecheii*, 2: *Tetragonisca angustula*; XO: Xanthine oxidase, DPPH: 2,2-diphenyl-1-picrylhydrazyl assay, TP: Total phenolic content, ORAC: Oxygen radical absorbance capacity assay; PMNs-LUM: Polymorphonuclear leucocytes-luminol, PMNs-CLA: Polymorphonuclear leucocytes-Cypridina luciferin analog

DISCUSSION

In Costa Rica, *T. angustula* followed by *M. beecheii* are the stingless bee species of most commercial interest. Their honey is the main product commercialized. Meliponini honey presents a blooming market due to an increased interest in its folk-attributed medicinal properties; among which, its use as a wound dressing is included [19,25]. A recent publication by Zamora *et al.* [20] reported that Costa Rican stingless bee honeys presented values of microbiological quality in accordance to European Pharmacopoeia's standards for non-sterile substances for pharmaceutical use and a broad-spectrum antimicrobial activity comparable to Medihoney®. In the present study, we decided to use Medihoney® as a reference since it is a FDA approved honey-based wound dressing and the only antioxidant therapy specifically approved for wound healing [11,12]. To our knowledge, this is the first study that compares Medihoney® and stingless bee honeys for their *in-vitro* antioxidant capacities and immunomodulatory activities.

An infected wound or a burn follows a Th2 type immune response, which is characterized by innate immunity [26-28]. The activation of the complement, coagulation, and the arachidonic acid cascades, in addition to the liberation of Th2 type cytokines promote the transendothelial migration of PMNLs to the affected tissue [29]. PMNLs will then recognize microorganisms via opsonins or lectins. Subsequently, the microorganisms will be engulfed in vacuoles, where, by means of the oxidative burst, the ROS produced will kill the microorganisms, to finally, be digested by proteolytic enzymes [30]. Hence, PMNLs are considered the first line of cell defense of innate immunity [31].

PMNLs and the ROS they produce, play vital roles in keeping homeostasis. They seek and destroy damaged cells and there is evidence that involves them with the facilitation of angiogenesis [32,33]. The ROS generated by PMNLs can act as signaling molecules that modulate crucial events like phagocytosis, gene expression, apoptosis, and epidermal growth factor-stimulated cell adhesion [34,35]. Although of utmost importance, excessive priming of PMNLs and the following liberation of ROS in the extracellular medium can severely damage healthy tissues [29,36]. ROS can damage macromolecules like proteins, DNA and can destroy cell membranes by lipid peroxidation [37,38]. Moreover, this oxidative stress delivers more free radicals like reactive nitrogen species and the activation of more inflammatory cells that perpetuate the inflammatory process (which is already a pro-oxidant condition); ironically complicates microbial infection and therefore, delays healing [26,28,34,36,39].

The basis of antioxidant therapy for wound healing is the interference of the signal transduction pathways of inflammation. A milestone that can be achieved by the administration of dressing agents with an antioxidant capacity that scavenge ROS, restore redox homeostasis by antagonizing oxidative stress, and consequently enhance wound healing [33,38,40].

We used the DPPH inhibition test for screening the radical quenching potentials of stingless bee honeys and Medihoney®. Our results suggest that special care should be taken while reaching a conclusion over the antioxidant capacity of honeys if such values are determined solely by the DPPH assay. Notwithstanding that most honeys inhibited DPPH in a dose-dependent fashion and the evident differences in the results according to bee species; in the present investigation the results for this assay did not correlate with the TP content and ORAC value. In addition, the positive correlation between the TP content and ORAC value reported herein, suggests that the antioxidant activity of the stingless bee honeys analyzed is given in a mayor grade due to their phenolic constituents.

The foremost ROS produced by PMNLs is the superoxide anion. In order to determine the scavenging activity of honeys specifically over superoxide, we performed a cell-free assay. Although the tests reported inhibition in a dose-dependent manner for all samples and the reference, these results are unreliable since all honeys and Medihoney® inhibited the enzyme XO. In the cell-free superoxide radical scavenging assay, the superoxide anion is generated as a product of the activity of XO over hypoxanthine [27,36].

In the wound environment, the main producers of superoxide are PMNLs. Although the superoxide generation by XO is considered by some authors redundant; this enzyme is expressed in the outer membrane of endothelial cells where its production of superoxide contributes to neutrophil recruitment and the generation of other more potent ROS via Fenton or Haber-Weiss reactions; all of which

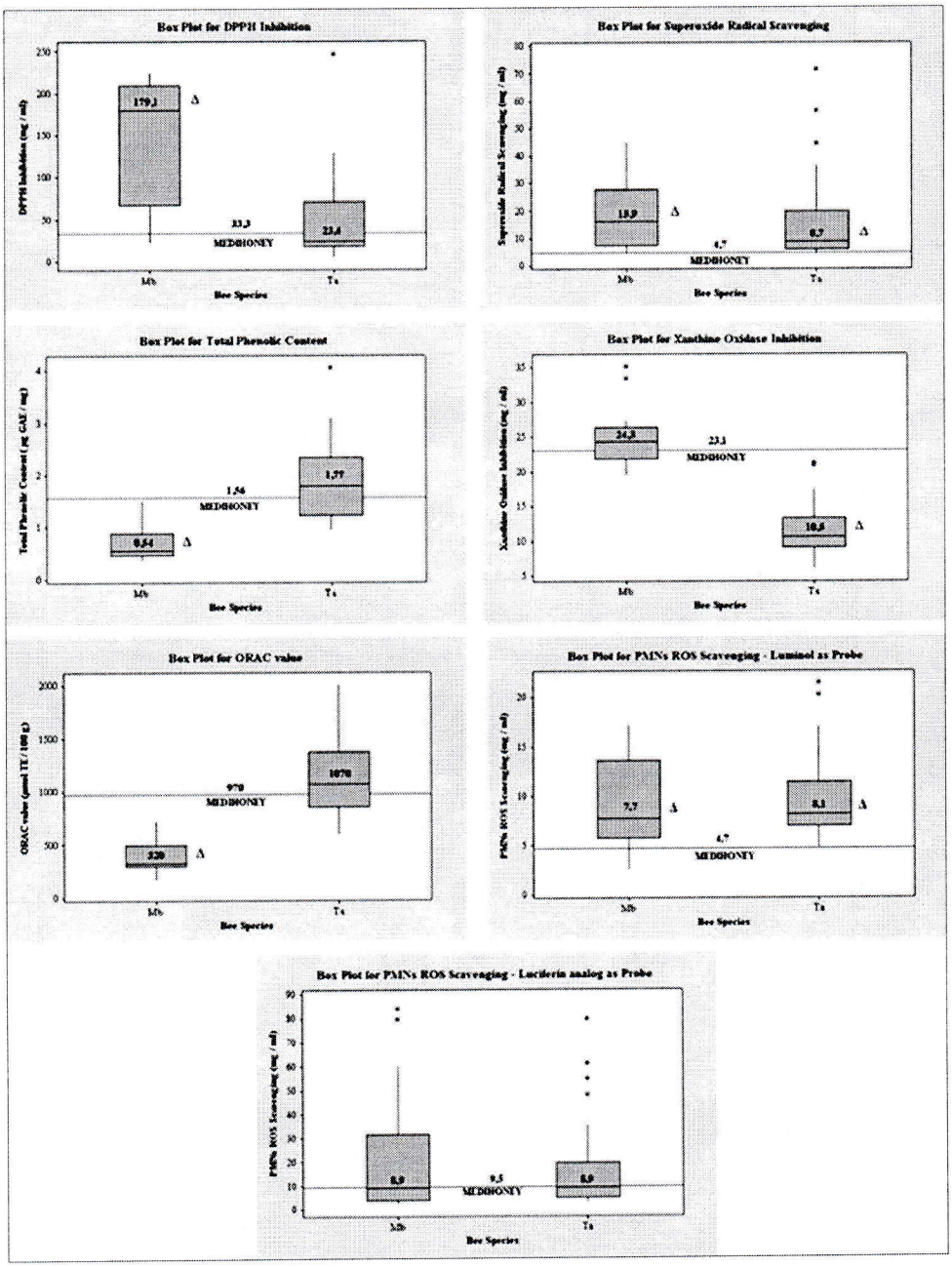

Figure 2: Boxplots for the results of the antioxidant capacity and immunomodulatory assays. The data are expressed as median values per bee species. The horizontal line in each graph indicates the results for Medihoney®; *atypical value; ᴬdifference between the median value and the reference result is statistically significant ($P < 0.001$)

enables a microvascular inflammatory response [34,36,41-43]. The inhibition of XO may not directly inhibit the generation of ROS by PMNLs [43], but a dose-dependent inhibitory activity over this enzyme may modulate the inflammatory response by affecting the ROS cascades and the neutrophil recruitment. The *T. angustula* honeys under study presented the highest activity over XO; a feature that correlates to its antioxidant capacity and unveils an adjuvant immunomodulatory action by means of inhibition of XO-mediated inflammatory responses.

The PMNL tests are antioxidant cell-based assays [22,44]. The chemiluminescence probe luminol detects the intra - and extracellular ROS, whereas the Cypridina luciferin analog solely detects ROS in the extracellular medium [39,45]. Medihoney® obtained the highest activity in the PMNLs test using luminol as luminescence probe, while the Meliponini honeys accomplished IC_{50} values in a close concentration range. Our results reported no statistical differences between Medihoney® and the stingless bee honey's performance in the PMNLs test using Cypridina luciferin analog as probe. The results delivered by both PMNLs experiments allow stating that the Meliponini honeys and the reference analyzed perform the function of radical scavengers in a similar fashion. The capability of all the honeys studied to render scavenging activity over ROS produced by

activated human polymorphonuclear leukocytes evidences an immunomodulatory activity by means of disrupting the ROS cascades; which impairs the inflammatory response and leads to redox homeostasis [33,38,39,40,46].

This is the first comparison of Meliponini honey and Medihoney® in their antioxidant capacities and immunomodulatory activities. Our study allows concluding that the *in-vitro* immunomodulatory activities of the honeys under study are given in a major grade due to radical scavenging and XO inhibition. Both mechanisms may play a role in the impairment of the inflammatory process and the promotion of redox homeostasis. The *T. angustula* honeys proceeding from Costa Rica presented antioxidant capacities not statistically different to Medihoney®. In addition, their radical scavenging activity over ROS produced by human PMNLs and inhibition of XO justify their ethnopharmacological use as a wound dressing and further research toward novel wound-healing developments.

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