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Radical Scavenging and Anti-Inflammatory Properties of Pectin from *Cissampelos pareira* Linn.

Nakuntwalai WISIDSRI[*] and Suradwadee THUNGMUNGMEE

Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi, Pathum Thani 12130, Thailand

([*]Corresponding author's e-mail: nakuntwalai_w@rmutt.ac.th)

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Abstract

Cissampelos pareira Linn. (*C. pareira*) has been used as a medicinal herb for treating fever and analgesic by Indian and Thai people. This experimental research investigates the scavenging ability of *C. pareira* pectin from leaves of variable concentrations on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) free radicals, its anti-inflammatory property on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells, and the cell viability. The experimental results show that the DPPH and NO scavenging performances of *C. pareira* pectin are positively correlated to the pectin concentrations, with corresponding half maximal inhibitory concentrations (IC_{50}) of 0.54 and 0.52 mg/ml. Meanwhile, the NO production in the LPS-stimulated macrophage cells is inversely correlated to the pectin concentrations. The cell viability in the LPS-stimulated macrophage cells is positively correlated to the *C. pareira* pectin concentrations, given the non-cytotoxicity of the extract compound. In essence, the inhibition of free radicals and the suppression of activated macrophages point to the usefulness of *C. pareira* pectin in functional dietary products and herb-based pharmaceuticals.

Keywords: *Cissampelos pareira* Linn., pectin, radical scavenging, anti-inflammation

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generate cellular signals and are beneficial to combating infectious agents. However, ROS/RNS are free radicals, with one unpaired electron derived from molecular oxygen or nitrogen. High concentrations of ROS/RNS induce oxidative stress and nitrosative stress and subsequent cell damage. Excessive free radicals, including ROS/RNS, induce oxidative stress, which in turn contributes to adverse cellular modifications, aging, and degenerative diseases, e.g., Parkinson's disease, arthritis, and arteriosclerosis, as well as cancer [1,2]. Inducible nitric oxide (NO) is an oxidative molecule generated by phagocytic cells, including macrophages. Excessive NO contributes to chronic inflammation and mediates most chronic diseases. Furthermore, the interaction between O_2^- and NO produces cytotoxic oxidant peroxynitrite ($ONOO^-$), which is a powerful oxidant to initiate lipid peroxidation and cleavage DNA, resulting in cancer [3].

An imbalance between oxidative stress and enzymatic/non-enzymatic antioxidants contributes to excessive ROS and RNS. Excessive free radicals seek stability by electron-pairing with the biological macromolecules of healthy cells, e.g., proteins, lipids, and DNA, resulting in damaged cellular proteins, lipids, and DNA and their impaired functions [2,4]. Endogenous and exogenous antioxidants play a role in protecting cells against free radicals and diseases. Both endogenous and exogenous antioxidants stabilize free radicals and break the oxidative chain. In addition, the antioxidants inhibit oxidation by scavenging free radicals [5]. Excessive free radicals and depleting antioxidants induce chronic inflammation and subsequent cell damage or cellular hyperplasia. In inflammation, ROS interact with DNA in mitotic cells, resulting in permanent genomic mutations, e.g., point mutations, gene deletions, or

gene rearrangements [6,7]. In addition, cellular antioxidant systems respond to excessive free radicals by activating genes involved in DNA renewal [8].

C. pareira is in the family of Menispermaceae. It is a tropical climbing plant ubiquitous in Asia, East Africa, and South America. The plant is traditionally used for treating skin disorders, abdominal pain, inflammation, dysentery, indigestion, and anorexia [9]. Furthermore, the ethanolic and methanolic extracts of *C. pareira* roots effectively scavenge ROS and RNS and prevent acute oxidative tissue injury in animal models [8,10]. The ethanolic extract of *C. pareira* roots also possesses anti-inflammatory properties in acute, subacute, and chronic inflammation, and antinociceptive and antiarthritic activities in rats [11,12]. The ethanolic extract of *C. pareira* (whole plant) exhibits analgesic and anti-inflammatory properties in rats [13]. In addition, the whole-plant methanolic extract of *C. pareira* is cytotoxic to the MCF-7 human breast cancer cell line [14].

The water extract of fresh *C. pareira* leaves forms gel in a short period of time at room temperature without the need for sucrose or calcium [15,16]. The main gelling ingredient of *C. pareira* leaves is low methoxyl pectin (LMP), consisting mainly of galacturonic acid and small amounts of neutral sugars [17]. In the food industry, pectin is used as an emulsifier, stabilizer, and thickener, as well as an ingredient in therapeutic diets, such as food gels for patients with diabetes mellitus.

Specifically, the aim of this research is to examine the scavenging ability of *C. pareira* pectin (leaves) of variable concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) free radicals, its anti-inflammatory property through inhibition of NO production in the lipopolysaccharide-stimulated RAW264.7 macrophage cells, and the cell viability, given the pectin concentrations of 0.05, 0.1, 0.25, 0.5 mg/ml.

Materials and methods

Extraction of *C. pareira* pectin

In this research, the *C. pareira* pectin extraction method was modified from [11], whereby *C. pareira* leaves from Thailand's Northern province of *Uttaradit* were first cleaned with water and dried at 45 ºC for 24 h. The dried leaves were ground and retained in vacuum at room temperature. The *C. pareira* dry powder was mixed in distilled water (1:50 w/v) and adjusted to pH 3.8 - 4.0 prior to heating in a water bath at 60 ºC for 60 min. The crude extract was filtered and centrifuged at 10000 rpm for 15 min. The supernatant was concentrated to half of its volume in a rotary evaporator prior to precipitation in ethanol 95 % (w/v) for the final concentration of 70 %. The precipitate was freeze-dried and ground for crude extract powder (i.e., *C. pareira* pectin). The powder was retained in vacuum for subsequent analysis.

Degree of esterification (DE) of *C. pareira* pectin

The methoxyl group of *C. pareira* pectin was determined in accordance with [16]. Specifically, 500 mg of pectin powder was first transferred to a 250 ml flask, followed by 2 ml of ethanol for moisture and 100 ml of carbon dioxide-free water to dissolve prior to adding 5 drops of phenolphthalein. The solution was titrated with 0.5 M sodium hydroxide for the initial titer. The solution (initial titer) was then added with 10 ml of 0.5 M sodium hydroxide, shaken vigorously, and allowed to stand for 15 min. Afterward, 0.5 M hydrochloric acid was added and shaken until the complete disappearance of the color pink. The resulting solution was mixed with 5 drops of phenolphthalein and titrated with 0.5 M sodium hydroxide, vigorously shaken, and terminated once a faint pink color was visible (end-point). The end-point served as the saponification titer (the final titer). The DE was calculated from the following formula;

$$\%DE = [\text{the final titer} / (\text{the initial titer} + \text{the final titer})] \times 100 \quad (1)$$

DPPH radical scavenging activity of *C. pareira pectin*

The *C. pareira* pectin powder was first dissolved in a pH 7.4 phosphate-buffered saline (PBS) solution by heating in a water bath at 60 °C. Approximately 75 µl of the dissolved pectin of variable concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) was mixed with 150 µl of 0.2 mmol/L DPPH (Sigma-Aldrich USA) solution (in methanol) and allowed to stand for 30 min without direct exposure to

light. The absorbance was determined at 517 nm using a micro-plate reader. In addition, PBS and L-ascorbic acid were used as the negative and positive controls, respectively. The DPPH scavenging capacity of the experimental pectin is expressed as a percentage of DPPH radical inhibition as below, where OD is the optical density;

%DPPH radical inhibition = [(OD without extract – OD with extract) / OD without extract) ×100 (2)

Nitric oxide (NO) radical scavenging of *C. pareira pectin*

In this research, sodium nitroprusside (SNP) (Sigma-Aldrich, USA) was utilized as the nitric oxide donor [18]. Specifically, 10 mM/L of SNP in a pH7.4 PBS solution was incubated with 1 ml dissolved *C. pareira* pectin of variable concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) at 25 ºC for 180 min. Approximately 100 µl of the resulting solution was withdrawn to react with a Griess Reagent kit (Promega, USA), whereby the solution was reacted with 20 µl sulfanilamide for 10 min and then 20 µl N-1-napthylethylenediamine dihydrochloride for another 10 min. The reaction mixture absorbance was measured at 560 nm and the nitric oxide (NO) concentrations were determined as the nitrite (NO_2^-) concentrations from the standard curve of a standard nitrite solution. PBS and L-ascorbic acid were used as the negative and positive controls, respectively. The NO scavenging capacity of the experimental pectin was expressed as a percentage of nitrite production inhibition using the following formula;

% NO_2^- production inhibition = [(NO_2^- without extract – NO_2^- with extract) / NO_2^- without extract] ×100 (3)

Cell culture

A RAW264.7 murine macrophage cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), containing 10 % fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, USA) at 37 °C in a humidified atmosphere of 5 % CO_2. The cells were subcultured twice a week. The cell viability was evaluated using 0.4 % trypan blue (Sigma-Aldrich, USA) with cell viability of 85 % as the minimum threshold.

NO production in the LPS-stimulated macrophage

The RAW264.7 macrophage cells (2×10^5 cell/ml) were pretreated with the dissolved *C. pareira* pectin of variable concentrations (0.05, 0.1, 0.25, 0.5 mg/ml) in a 96-well plate and incubated at 37 °C for 24 h. The pre-treated cells were stimulated with 1 µg/ml of lipopolysaccharides (LPS) and incubated for another 24 h. The NO concentrations were determined from nitrite (NO_2^-) in the stimulated-cell supernatant using a Griess reagent kit, whereby 100 µl of the supernatant was reacted with 20 µl sulfanilamide for 10 min and with 20 µl N-1-napthylethylenediamine dihydrochoride for another 10 min. The reaction mixture absorbance was measured at 560 nm and the nitric oxide (NO) concentrations were determined as the nitrite (NO_2^-) concentrations from the standard curve of a standard nitrite solution. PBS (pH 7.4) and 100 µM of dexamethasone each with 1 µg/ml LPS were respectively used as the negative and positive controls.

Cell viability of the LPS-stimulated macrophage

The viability of the residual macrophage cells after the NO assay, given the *C. pareira* pectin concentrations of 0.05, 0.1, 0.25, 0.5 mg/ml, was determined by resazurin (Sigma-Aldrich, USA) reduction assay, whereby the residual cells were incubated for 2 h at 37 °C in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 against 600 nm.

Statistical analysis

In this research, the experiments encompass the DPPH and NO scavenging ability, given the *C. pareira* pectin concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml, and the NO production and cell viability, given the pectin concentrations of 0.05, 0.1, 0.25, 0.5 mg/ml. Each of the experiments was carried out in triplicate and then repeated in triplicate for a total of 198 treatments. The statistical data

were expressed as means ± standard error of mean (SEM). The negative control (PBS) group were compared against the experimental group using one-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test, with 5 % ($p < 0.05$) and 1 % ($p < 0.01$) significance levels.

Results and discussion

C. pareira pectin and degree of esterification

The yield of pectin from *C. pareira* leaves relative to the dried weight of *C. pareira* leaves was 3 %, with the degree of esterification (DE) of 42.22 %. The experimental *C. pareira* pectin was of low methoxyl pectin (LMP), given that a DE below 50 % is regarded as low methoxyl pectin.

DPPH scavenging capacity of *C. pareira* pectin

In the course of the DPPH radical scavenging by plant extracts, the color of DPPH alters from purple to yellow [1,19]. In this research, the DPPH scavenging capacity of *C. pareira* pectin is determined by the percentage of DPPH free radical inhibition and half maximal inhibitory concentration (IC_{50}). IC_{50} is the inhibitory concentration (IC) at which the DPPH radicals are scavenged by 50 %. The results revealed that, with 0.125 - 5 mg/ml (0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) pectin concentrations, *C. pareira* pectin significantly reduces DPPH, achieving the inhibition performance in the range of 7.32±3.73 - 82.17±0.55 % (**Figure 1**); and an IC_{50} of 0.54 mg/ml (**Table 1**). Meanwhile, L-ascorbic acid (0.5 mg/ml), which was used as the positive control, exhibits the inhibition efficiency of 90.91±0.16 %.

Nitric oxide scavenging capacity of *C. pareira* pectin

In general, scavengers of nitric oxide (NO) compete with oxygen and reduce NO production [18]. The experimental results revealed that *C. pareira* pectin could scavenge the NO radicals. In the absence of *C. pareira* pectin, the negative control (PBS) exhibited a high NO_2^- concentration, while L-ascorbic acid (the positive control) inhibited nitrite (NO_2^-) formation. The results revealed that, with 0.0625 - 5 mg/ml (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) pectin concentrations, *C. pareira* pectin significantly reduces NO_2^-, achieving scavenging performance in the range of 11.86±2.4 - 95.88±0.94% (**Figure 1**), and an IC_{50} of 0.52 mg/ml (**Table 1**), where IC_{50} is the inhibitory concentration (IC) at which NO radicals are scavenged by 50 %. By comparison, L-ascorbic acid (the positive control) at 0.5 mg/ml achieved inhibition performance of 98.22±0.85 %.

Table 1 DPPH and NO half maximal inhibitory concentrations (IC_{50}) of *C. pareira* pectin and L-ascorbic acid.

Compound	IC_{50} of DPPH	IC_{50} of NO
C. pareira pectin	0.54 mg/ml	0.52 mg/ml
L-ascorbic acid	0.027 mg/ml	0.072 mg/ml

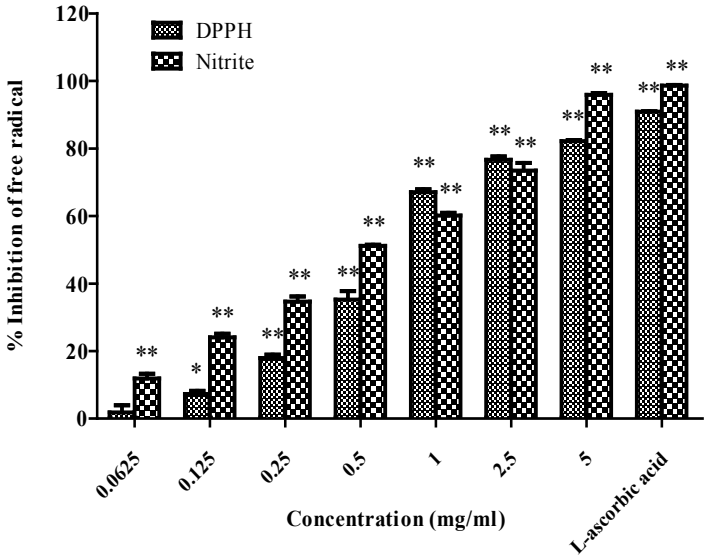

Figure 1 DPPH and NO (nitrite) inhibition efficiency (%) under variable pectin concentrations, compared with pectin-free PBS (negative control). Values are means ± SEM. * and ** respectively denote $p < 0.05$ and $p < 0.01$. L-ascorbic acid is used as positive control.

Effect of *C. pareira* pectin on nitric oxide production in LPS-stimulated macrophage

In this research, the RAW264.7 macrophage cells were treated with *C. pareira* pectin of variable concentrations (0.05, 0.1, 0.25, 0.5 mg/ml) for 24 h prior to stimulation with 1 µg/ml LPS. In addition, PBS and 100 µM of dexamethasone, each with 1 µg/ml LPS, were respectively used as the negative and positive controls. In **Figure 2(a)**, the experimental results revealed that the nitrite concentrations in the LPS-stimulated macrophage were inversely correlated to the *C. pareira* pectin concentrations. NO inhibition was more pronounced in the treatments with 0.1 - 0.5 mg/ml pectin concentrations. The PBS-treated cells (negative control) exhibited very high nitrite concentrations, while the dexamethasone-treated cells (positive control) achieved high NO inhibition performance, as evidenced by low nitrite concentrations.

Effect of *C. pareira* pectin on cell viability

The viability of the RAW264.7 macrophage cells was assessed by a resazurin reduction assay and the effective pectin concentrations determined. PBS and dexamethasone, each with 1 µg/ml LPS, were respectively used as the negative and positive controls. In general, cell viability and NO production were positively correlated. Given the non-cytotoxicity of *C. pareira* pectin, the pectin-treated cells could achieve high NO inhibition performance (lower nitrite concentrations) with elevated cell viability. In **Figure 2(b)**, the results indicated that the cell viability in the LPS-stimulated macrophage was positively correlated to the *C. pareira* pectin concentrations. In fact, the cell viability of the pectin-treated cells was comparable to that of the PBS- (negative control) and dexamethasone-treated cells (positive control).

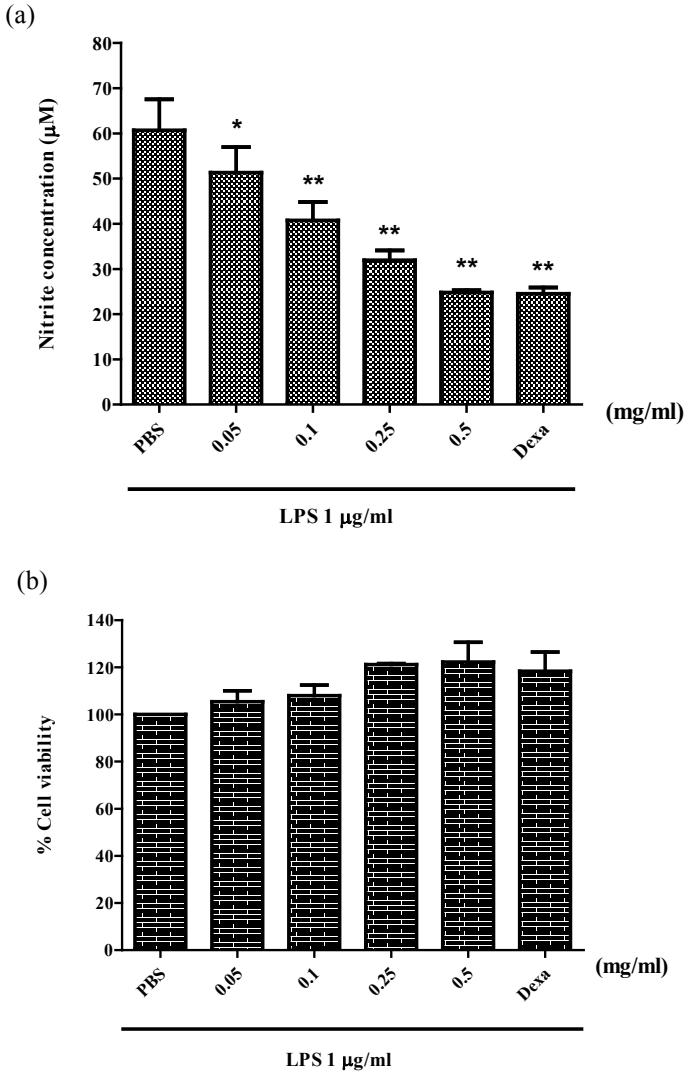

Figure 2 Effects of variable pectin concentrations in 1µg/ml LPS-stimulated RAW264.7 macrophage: (a) nitrite concentrations (NO production), (b) cell viability. Values are means ± SEM. * and ** respectively denote $p < 0.05$ and $p < 0.01$. PBS and Dexamethasone are used as negative and positive controls.

Discussion

Pectin products are classified by the degree of methyl-esterification into high and low methoxyl pectin. An esterification degree (DE) above 50 % denotes high methoxyl pectin (HMP) and that of below 50 % low methoxyl pectin (LMP). In this research, the degree of esterification (DE) of *C. pareira* pectin was 42.22 % and, thus, was of low methoxyl pectin (LMP). This is consistent with [16], who documented that the structure of *C. pareira* pectin is a 1,4-linked chain of poly-α-D-galacturonic acid, with 41.7 % DE.

The experimental results also demonstrate the ability of *C. pareira* pectin in scavenging DPPH and NO free radicals. The free-radical scavenging capacity of *C. pareira* pectin is attributable to electron-donating compounds that react with free radicals to stabilize and inhibit radical chain reaction. Previous

research reported the DPPH and NO scavenging ability of plant-based pectins, e.g., pectin from the husk of *Cicer arietinum* L. [20] and citrus [21], and apple cultivars from central Europe [22]. Likewise, this current research shows that *C. pareira* pectin from *C. pareira* leaves possesses antioxidant properties.

Oxidative stress contributes to inflammatory diseases and disorders. Under excessive stress, the body produces large amounts of ROS. If the endogenous enzymatic and non-enzymatic antioxidants fail to cope with the excessive ROS, antioxidant imbalance and cell damage results. Furthermore, exogenous antioxidant deficiency from daily diet leads to the development of degenerative diseases and inflammatory illnesses [23]. In fact, exogenous antioxidants from food and other dietary sources play a crucial role in combating free radical formation, neutralizing existing free radicals, and repairing damaged molecules, as well as interacting with endogenous antioxidants to enhance antioxidation capacity [24].

Specifically, *C. pariera* pectin is safe for consumption, and could be deployed as an exogenous antioxidant functioning as a free-radical scavenger. In Singthong *et al*. [16], *C. pareira* pectin is of low methoxyl pectin, with its crude extract consisting mainly of uronic (galacturonic) acid (70.56 %), with an average molecular weight of 55 kDa and small quantities of neutral sugars. The effective antioxidant activity of *C. pareira* pectin could be attributed to the high galacturonic acid content. In Chen *et al*. [25], high contents of galacturonic acid and relatively low-molecular-weight polysaccharide enhance antioxidant activity; nevertheless, the mechanism of polysaccharide is not yet fully understood.

In addition, this current research portrays the anti-inflammatory property of *C. pareira* pectin through inhibition of NO production in LPS-stimulated macrophages. NO is a signaling molecule involved in the physiological and pathological processes of the body. Excessive NO induces a pathologically cytotoxic environment and inflammatory disorders [26], indicating that NO-induced oxidative stress is closely linked with inflammation.

Specifically, NF-κB is a key transcriptional factor linking oxidative stress and inflammation. Oxidative stress activates a primary disorder and NF-κB to generate inflammation, which progresses as a secondary disorder and further aggravates oxidative stress. On the other hand, inflammation as a primary disorder induces oxidative stress as a secondary disorder, which further exacerbates inflammation [27]. The free-radical scavenging and anti-inflammatory properties of *C. pareira* pectin could, thus, possibly be attributed to its NF-κB inhibitory property.

In Chen *et al.* [28], citrus pectins with DE30, DE60, and DE90 effectively inhibit the NO-generating enzyme, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in LPS-stimulated macrophages [28]. In an animal model, administration of modified LM and HM citrus pectins hinders the progression of colitis in mice. In Popov *et al.* [29], the LM pectin decreases pro-inflammatory cytokine and tumor necrosis factor-α (TNF-α), and improves the survival rate in mice that received a lethal dose of LPS.

In Popov *et al.* [30], pectic polysaccharide from fresh plum (*Prunus domestica* L.) reduced the adhesion of peritoneal leukocytes and inhibited superoxide anion radical production by reducing xanthine oxidase activity. Furthermore, administration of pectin from berries of cowberry (*Vaccinium vitis-idaea* L.) reduced intestinal wall lesion and myeloperoxidase (MPO) activity in mice [31]. In this current research, *C. pareira* pectin from leaves effectively inhibits NO production in macrophage cells and, thus, the cellular anti-inflammatory mechanism.

Furthermore, pectin is used as a food emulsifier, stabilizer, and thickener. Different pectin structures lead to different gelling properties, emulsion activities, emulsion stabilities, and releasing properties in complex food matrices. Pectin is also used in jellies, bakery fillings, and potable milk [32]. Furthermore, pectin is a functional food for lowering blood cholesterol and prolonging gastric emptying, as well as probiotic delivery and food preservation [33]. The LM pectin improves the color stability and bioactive properties of blackberry jam, in addition to elevated antioxidant functions [34].

In pharmaceuticals, pectin is used as a drug carrier for controlled drug release, including nasal, oral, and ocular drug delivery, as well as cancer-targeted drug delivery, polyplexes for gene therapy, and wound healing patches [35]. Given the low DE of *C. pareira* pectin (LM pectin) and its effective antioxidant and anti-inflammatory properties, the extract compound (*C. pareira* pectin) could, thus, be applied to enhance food attributes and develop novel pharmaceuticals. In light of the NF-κB inhibitory

property of *C. pareira* pectin, further research would focus on its *molecular* anti-inflammatory mechanism and subsequent application in innovative dietary products and advanced pharmaceuticals.

Conclusions

This experimental research has investigated the scavenging ability of *C. pareira* pectin of variable concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5 mg/ml) on DPPH and NO free radicals, its anti-inflammatory property through inhibition of NO production in LPS-stimulated RAW264.7 macrophage cells, and the cell viability, given pectin concentrations of 0.05, 0.1, 0.25, 0.5 mg/ml. The experimental results revealed that the DPPH and NO scavenging performance of *C. pareira* pectin and the compound concentration are positively correlated, with corresponding half maximal inhibitory concentrations (IC_{50}) of 0.54 and 0.52 mg/ml. Moreover, NO production (presented as nitrite concentrations) in the LPS-stimulated macrophage cells is inversely correlated to *C. pareira* pectin concentrations. Cell viability in the LPS-stimulated macrophage is positively correlated to *C. pareira* pectin concentrations, given the non-cytotoxicity of the extract compound. The findings demonstrate the usefulness of *C. pareira* pectin in a multiple of industry applications, including foods, cosmetics, and pharmaceuticals.

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