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Valorization of coffee bean processing waste for the sustainable extraction of biologically active pectin

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ABSTRACT

The dry method of coffee processing generates a significant amount of coffee husk, an agricultural waste for which currently there is a lack of profitable use, and their disposal constitutes a major environmental problem. Pectin was extracted from coffee husk using citric acid solution (pH 1.5) by microwave-assisted extraction method, followed by using ice-cold ethanol. The coffee husk pectin (CHP) with a yield of 40.2% was characterized using SEM, FT-IR, and XRD techniques. The CHP exhibited significant *in-vitro* antioxidant activity as measured by using 2,2-diphenyl-1-picryl-hydrazyl; (IC_{50} value of 395.1 ± 0.42 μg/mL), ferrous reducing antioxidant capacity ($A_{700\ nm}$ = 0.55 ± 0.08), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (42.02 ± 0.38%) and ascorbic acid auto-oxidation inhibition (92.01 ± 0.28%) assays. CHP demonstrated antibacterial activity against *Escherichia coli* and *Bacillus cereus* with an inhibition diameter of 20 ± 1.01 mm and 18 ± 0.84 mm, respectively. Interestingly, CHP showed a significant anti-inflammatory effect by negatively modulating the expressions of TNF-α and TGF- β in LPS-stimulated macrophage cell lines. Collectively, our findings suggest that the coffee husk is a potential source of commercial pectin, microwave-assisted extraction has a great potency on the commercial pectin extraction from the coffee husk and CHP demonstrates significant biological activity.

1. Introduction

A drastic increase in population coupled with urban industrialization and enhanced food production has accelerated the generation and accumulation of various waste materials [1]. The agricultural by-products and wastes are causing a series of environmental problems, by contaminating surface and groundwater, generation of off-odors, methane release by anaerobic waste decomposition of waste, and alteration in soil pH and microbiome (Ayilara et al., 2019). However, these agricultural by-products and wastes have the

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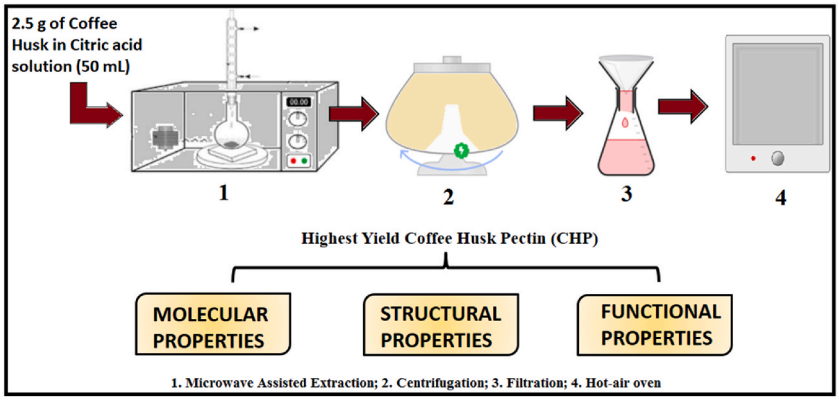

Fig. 1. Overall scheme followed in the study for extracting pectin from coffee husk and evaluating its bioactivity.

potential to be recycled and can be exploited for value-added products production *viz.,* enzymes, food additives, organic acids, and biofuels [2].

Globally India accompanies 8th place among the highest coffee-producing countries and produced 312 metric tons of coffee in 2020 (International Coffee Organization, 2020). The dry method of coffee bean processing produces a significant amount of organic biological material *i.e.,* coffee husk (coffee seed coat), which is currently being underutilized [3]. This is generally discarded as landfill mass, mixed with animal fodder, or incinerated. Due to their higher level of bioactive compounds *viz.*, tannins, caffeine, and trigonelline, disposal of coffee husks directly into the environment can degrade the soil quality and when mixed with animal fodder might induce carcinogenicity [4]. Approximately 1 ton of coffee husk is generated for every ton of coffee beans processed [5]. Thus, in compliance with the perception of sustainable development, innovative techniques, and products for profitable use, many researchers across the globe are attempting to use coffee husk for the production and extraction of value-added products. Coffee husk was used as a source to extract phenolic compounds [6]; bioactive compounds [7], for the production of succinic acid [8], for extracting cellulose and cellulose nanocrystals [9]. In addition, coffee husk was used as a biosorbents for the removal of heavy metal ions (Pb^{2+} and Cd^{2+}) and dye (malachite green and methylene blue) from wastewater [10–12] and the fermentation production of mosquitocidal bacteria [13]. Proximate analysis by Ref. [3] reported that the coffee husk comprises majorly carbohydrates (16–85%) followed by proteins (7–17%) and lipids (0.5–3%). Pectin is found to be the tentatively identified polysaccharide in the coffee husk [3], and thus advancements in the field of industrial biotechnology paves way for the economic exploitation of coffee husk as a source of pectin. Many research groups across the globe are now actively using coffee husk, pulp and mucligae as a source of pectin [2,14,15]; June G 1986). However, to the author's knowledge, this is the first article that describes the use of coffee husk (Robusta) as a source of pectin and evlauting its biological properties.

The extraction technique plays a vital role in the successful isolation of bioactive compounds from natural sources [16]. Numerous extraction techniques have been applied to extract pectin from different plant sources *viz.,* apple [17], citrus peel [18], carrots [19], sugar beet pulp [20], sunflower heads (Tan et al., 2021), papaya [21], the coffee pulp [22]. Conventional and chemical extraction techniques may pose serious environmental problems as they generate acidified wastewater [23,24]. Microwave-assisted extraction appears to be an innovative and advanced technique, developed as a substitute for available traditional methods [25]. This technique is advantageous from short processing time and low energy consumption, low solvent requirements and low cost, easy controllability, and high efficiency [26].

In recent times, research efforts are focused on evaluating the biological properties of pectin due to its diverse applications in the healthcare, food, and cosmetic industries [27]. Previous research findings have shown that the presence of galacturonic acids in the pectin backbone with several electron-donating groups (methoxy, carboxyl, and hydroxyl groups) contributes to its antioxidant activity [28]. In addition, pectin has demonstrated *in vitro* antibacterial and anti-inflammatory properties [7,29]. Antioxidant and anti-glycosylation activities of pectin extracted from coffee husk (Arabica) is demonstrated by Ref. [14]. However, limited information is available on the antioxidant, antibacterial, and anti-inflammatory properties of pectin obtained from coffee husk (Robusta). In line with this, the present investigation aims to cover this gap by evaluating the effectiveness of coffee husk as a source of pectin. Apparently, no studies discussed the characterization of pectin from coffee husk along with its biological activity. Therefore, extracted CHP was characterized using suitable techniques and evaluated its biological properties *viz.,* antioxidant, antibacterial, and anti-inflammatory properties (Fig. 1). Furthermore, the present study paves way for understanding the relationship between the chemical structure and biological activity of CHP to expand their use in pharmacology and medicine.

2. Materials and methods

2.1. Materials

Coffee husk from coffee beans (Robusta) was obtained from coffee processing plants in Chikmagalur, Karnataka, India (13.3143° N,

75.7710° E). Pectin standard, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were procured from Sigma-Aldrich (USA). Phenolphthalein, ethanol, and citric acid were obtained from Sisco Research Laboratories (India). Other chemicals and reagents used in the study were of analytical grade.

2.2. Microwave-assisted extraction of pectin from coffee husk

Pectin extraction was performed as described by Ref. [30] using NuWav-Pro Microwave Reactor System (Nutech Analytical Technologies Pvt. Ltd., India) at a working frequency of 2450 MHz with adjustable irradiation time and microwave power (maximum power output: 1000 W) under different microwave-assisted extraction conditions. The effects of four independent parameters *viz.*, microwave power (100, 275, 450, 625 and 800 W), irradiation time (5, 10, 15, 20 and 26 min), temperature (50, 62.5, 75, 87.5 and 100 °C) and solid/solvent ration (2.5, 5, 7.5, 10 and 12.5 g/50 mL) at five different levels for were studied through Central Composite Rotatable Designs (CCRD) using Design-Expert V10 statistical software (unpublished data). The optimum condition of the selected process parameters to obtain maximum CHP was found to be microwave power of 450 W, temperature of 75 °C, solid/solvent content of 2.5 g coffee husk/50 mL citric acid solution, and extraction time of 15 min. A 250 mL quartz round bottom flask containing the desired solid/solvent ratio was placed in the middle of the microwave reactor over a rotating dish and subsequently exposed to different microwave power and irradiation time. After the extraction process, a mixture in the quartz flask was allowed to cool down to room temperature, and centrifugation at 6000 rpm for 15 min was performed to collect the supernatant. The supernatant was treated with equal volumes of 95% ethanol, the coagulated CHP was subsequently washed three times using ethanol and then recovered using a muslin cloth. Wet CHP was subjected to drying at 50 °C in a hot air oven until it attains a constant weight. The CHP yield (%) was calculated using the below equation (1) [31].

$$\textbf{CHP yield} = \frac{\text{Weight of the dried CHP (g)}}{\text{Weight of coffee husk used for extraction (g)}} \text{ x } 100 \tag{1}$$

2.3. Characterization of CHP

Morphological and structural characteristics of the extracted pectin were studied using SEM (Carl Zeiss- ULTRA 55, Germany) at different magnifications. An FT-IR spectrum was studied using an FT-IR instrument as described by Ref. [32]. The transmittance spectra were recorded in the range of 4000–500 cm^{-1} wavenumbers at a resolution of 4 cm^{-1}. The crystal structure was observed by an X-ray diffractometer (RigakuUltima IV, Japan). Herein, the scanning range was from 5 to 50°, and the scanning rate was 5°/min.

2.4. Determination of molecular properties

Moisture and ash content was determined using 0.5 g of extracted pectin by gravimetric method [5]. The equivalent weight was determined as described by Ref. [33]. Briefly, CHP (0.25 g) moistened with ethanol (2.5 mL) was made up to 100 mL using distilled water. NaCl (1 g) and phenolphthalein indicator (6 drops) were added to the mixture and titrated against 0.1 N NaOH until the indicator color changed to pink (pH 7.5). The equivalent weight is calculated from **equation (2)**.

$$\textbf{\textit{Equivalent weight}} = \frac{\text{Weight of pectin (mg)}}{\text{Volume of NaOH (mL)} * \text{Normality of NaOH}} \tag{2}$$

To determine the methoxyl content, to the above obtained neutralized solution, 0.25 N NaOH (12.5 mL) was added, the solution was then shaken vigorously, and allowed to stand at room temperature for 30 min in a closed Erlenmeyer flask. 0.25 N HCl (12.5 mL) and the phenolphthalein indicator were added and titrated with 0.1 N NaOH until the solution turned pink. The Methoxyl level is calculated using **equation (3)** [33].

$$\textbf{\textit{Methoxyl level}}\ (\%) = \frac{\text{Volume of NaOH (mL)} * 31 * \textit{Normality of NaOH} * 100}{\text{Weight of CHP (mg)}} \tag{3}$$

where, 31 is molecular weight of methoxyl in the form of CH_3O

Total anhydrouronic acid (AUA) content in the extracted CHP was quantified as described by Ref. [9] using **equation (4)**.

$$\textbf{\textit{AUA}}\ (\%) = \frac{(0.1Z + 0.1Y) * \ 176 * 100}{\text{Weight of CHP (mg)}} \tag{4}$$

wherein the molecular unit of AUA (1 Unit) is 176 g; Z = titer mL of NaOH from equivalent weight determination and Y = titer mL of NaOH from methoxyl level determination.

The degree of esterification (DE) of the extracted CHP was measured based on the AUA (%) and methoxyl level using **equation (5)**.

$$\textbf{\textit{DE}}\ (\%) = \frac{176 * \%\ \text{Methoxyl level}}{31 * \%\ \text{AUA}} * 100 \tag{5}$$

where, 31 is molecular weight of methoxyl in the form of CH_3O and and 176 is the molecular weight of galacturonic acid.

2.5. Evaluation of functional properties

2.5.1. Antioxidant activity of CHP

2.5.1.1. DPPH radical scavenging activity. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging ability, an indicator of hydrogen-donating ability, was measured [34]. Ascorbic acid was used as the reference standard. The antioxidant activity was carried out with varying concentrations of ascorbic acid and CHP [33]. The varying concentration of standard and CHP was made to react with 40 μg/mL of DPPH solution and the IC_{50} values were calculated from the linear regression curve and using **equation (6)**.

$$\%\ \boldsymbol{DPPH\ scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} * 100 \tag{6}$$

2.5.1.2. Ferrous reducing antioxidant capacity (FRAC) assay. Ferrous reducing antioxidant capacity (FRAC) of the CHP was determined as described earlier [35]. Varying concentration of ascorbic acid as standard (0.25 mL; 12.5–150 μg/mL) or CHP (0.5–10 mg/mL) was made to react with 1% potassium ferricyanide, $[K_3Fe\ (CN)_6]$ solution (0.625 mL) and potassium buffer (0.2 M; 0.625 mL) at 50 °C for 20 min. Then trichloro acetic acid (TCA; 10%; 0.625 mL) solution was added and the reaction mixture was centrifuged for 10 min at 3000 rpm. The supernatant (1.8 mL) was mixed with distilled water (1.8 mL) and ferric chloride ($FeCl_3$; 0.1%; 0.36 mL) solution. The absorbance of the mixture was read at 700 nm. Increased absorbance of the reaction mixture indicates increased ferrous-reducing ability [36].

2.5.1.3. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assay. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assay was performed as described by Ref. [37]. In brief, aliquots of varying CHP concentration (150 μl) was treated with ABTS solution (7.4 mM; 2.85 mL) and incubated for 10 min at room temperature. The absorbance of the sample was read at 734 nm and the % ABTS free radical scavenging was calculated using the below **equation (7)**.

$$\%\ ABTS\ free\ radical\ scavenging = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} * 100 \tag{7}$$

2.5.1.4. Ascorbic acid auto-oxidation inhibition. Varying concentrations of CHP (0.2 mL) were mixed with ascorbic acid solution (5 mM; 0.1 mL) and phosphate buffer (0.2 M; pH 7; 9.7 mL). The mixture was incubated at 37 °C for 10 min and absorbance was read at 265 nm. The % of ascorbic acid auto-oxidation inhibition was calculated using **equation 8** [34].

$$Inhibition\ of\ ascorbic\ acid\ auto-oxidation\ (\%) = \left[\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} - 1\right] * 100 \tag{8}$$

2.5.2. Antibacterial activity

Antibacterial activity was evaluated against *E. coli* and *B. cereus* by the agar well diffusion method [29]. Briefly, a bacterial suspension (10^7 cells/mL) was spread onto the Luria Bertani Agar. Wells were made upon solidification using a sterile cork borer (6 mm in diameter). CHP (100 μL) of varying concentrations prepared using sterile water was added to respective wells. The plates were refrigerated (30 min) to facilitate the diffusion of CHP from the well into the agar [38]. Further, plates were incubated at 37 °C for 24 h and the activity was evaluated as growth inhibition halos around each spot.

2.5.3. In-vitro cytotoxicity using MTT assay

The MTT assay was used to assess whether the CHP is cytotoxic to RAW264.7 cell lines (NCCS, Pune, India). The RAW 264.7 cells cultured in DMEM medium supplemented with 1 0% FBS, 1% antibiotic-antimycotic solution, and 1% L-glutamine wereincubated at 37 °C in 5% CO_2. To determine cell viability, RAW 264.7 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well, allowed to grow for 24 h. The cells were treated with LPS (1 μg/mL) for 4 h, followed by treatment with varying concentrations of CHP (12.5–200 μg/mL), and incubated for 24 h. Cell viability was determined by MTT assay The cell morphology was examined using an inverted biological microscope (Biolink, India). The percentage of cell viability was calculated using **equation (9)**.

$$\%\ \text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} * 100 \tag{9}$$

2.5.4. Anti-inflammatory activity using flow cytometry

The anti-inflammatory activity of CHP was assayed using LPS-stimulated RAW264.7 cells. Flow cytometry with FACS Calibur (BD Biosciences, CA, USA) was used to determine the % cells expressing TNF-α and TGF-β intensity under various experimental conditions. In summary, cells in a 6-well plate at a density of 2.5×10^5 cells/mL were incubated for 24 h. Then, the cells were treated with LPS (1 μg/mL) as an inflammatory stimulus for 4 h, followed by treatment with CHP (50 μg/mL), and further incubated for 24 h. LPS-treated cells were used as a positive control, untreated cells were used as a negative control, and the cells treated with a synthetic non-steroidal anti-inflammatory drug, diclofenac (35 μg/mL) was used as a standard control. At the end of the treatment, cells were fixed using 70% ethanol, and the % of cells expressing TNF-α and TGF-β were quantified using specific antibodies *viz.,* fluorescein isothiocyanate (FITC) and Phycoerythrin (PE) antibodies (BD Biosciences, CA, USA), respectively. The results were analyzed using BD Cell Quest Pro version

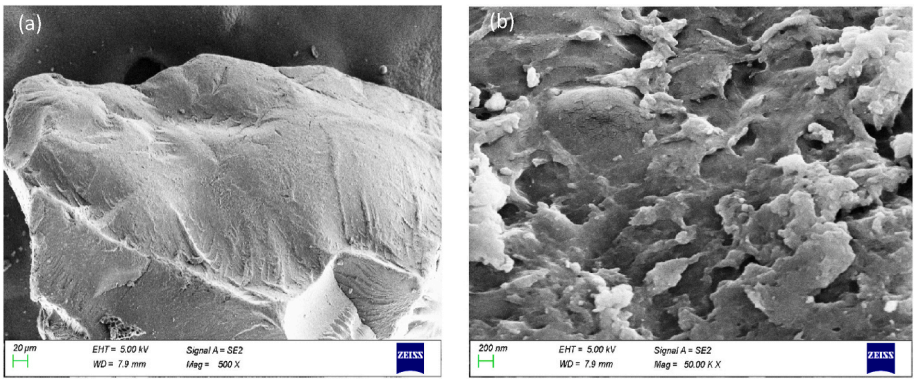

Fig. 2. Scanning Electron Microscope (SEM) images of Coffee Husk Pectin (CHP) at different magnifications and resolutions (a) 500× and 20 µm (b) 50 KX and 200 nm.

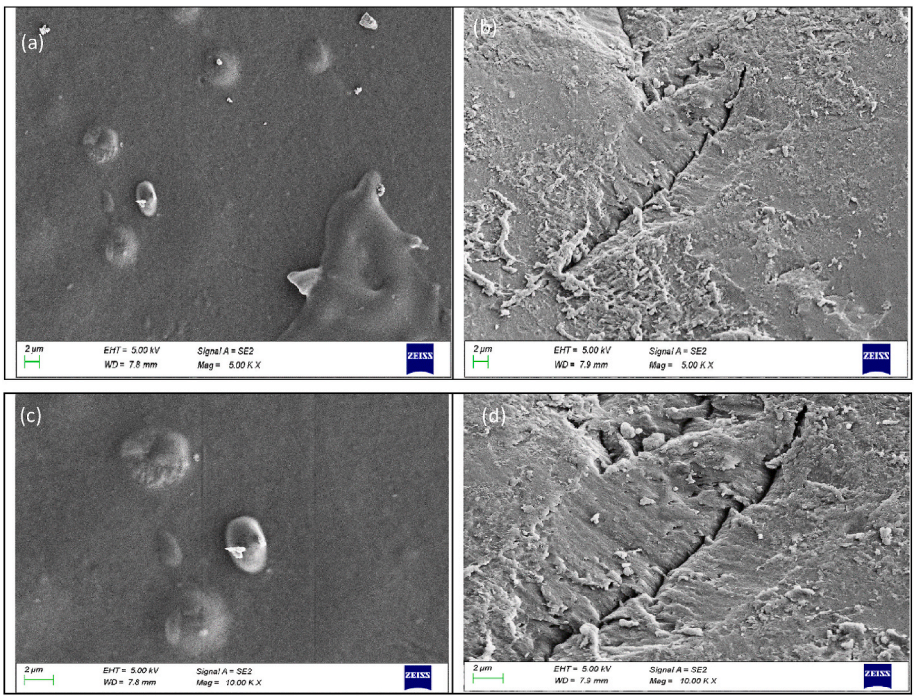

Fig. 3. Scanning Electron Microscope (SEM) images commercial pectin and Coffee Husk Pectin (CHP) at same resolution and magnification (a) Commercial pectin: 5 KX and 2 µm (b) Coffee Husk Pectin (CHP): 5 KX and and 2 µm (c) Commercial pectin: 10 KX and 2 µm (d) Coffee Husk Pectin (CHP): 10 KX and and 2 µm.

6.0 software.

2.6. Statistical data analysis

All the experiments were performed in triplicates. The experimental data obtained were expressed as Mean ± SD. Statistical significance was analyzed by one-way ANOVA followed by a post-hoc Dunnett test. Statistical analysis was performed using InStat3 software (v3.36).

3. Results and discussion

3.1. Microwave-assisted extraction of CHP

Mass transfer of pectin into the solvent during the extraction from the lignocellulosic biomass significantly influence by the

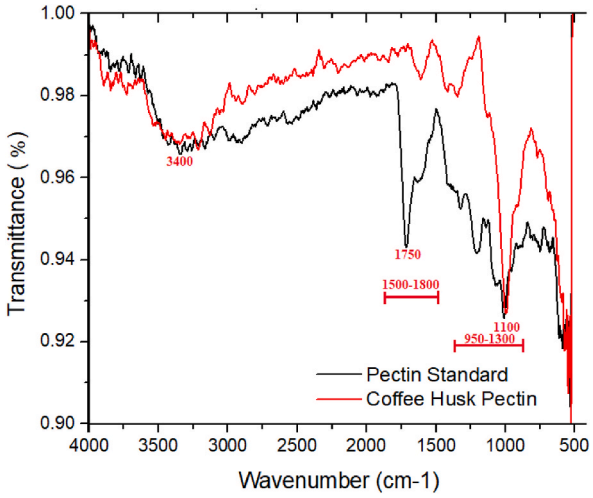

Fig. 4. FT-IR spectrum of Standard Pectin and Coffee Husk Pectin (CHP).

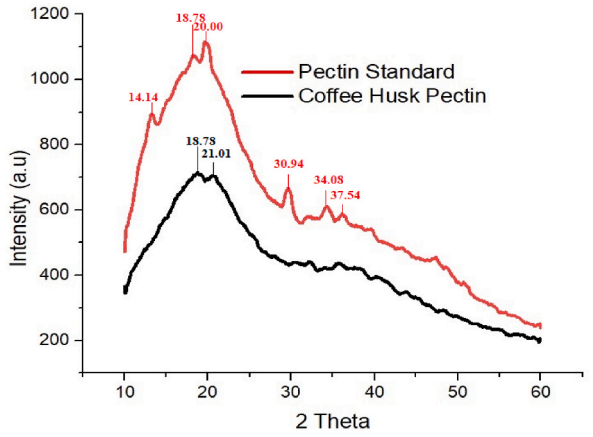

Fig. 5. X-Ray Diffraction patterns of Standard Pectin and Coffee Husk Pectin (CHP).

extraction method. Among the various methods of pectin extraction, microwave-assisted extraction gaining popularity due to its shorter extraction time, less solvent consumption with higher and better quality of extracted pectin [39]. Due to the strong formation of vapor in polar substances created by the electromagnetic field, microwave-assisted extraction is reported to be more effective than other extraction methods [40]. Furthermore, irradiation of raw materials using a microwave is reported to enhance pectin yield by allowing greater structural disintegration [41]. The CCRD matrix employed to optimize the process parameters revealed optimum conditions were microwave power of 450 W, temperature of 75 °C, solid/solvent content of 2.5 g coffee husk/50 mL citric acid solution, and extraction time of 15 min. The CHP yield of 40.2% was obtained.

3.2. Characterization of CHP

SEM analysis was performed to analyze and characterize the surface morphology of the CHP (Fig. 2). The structure of the pectin is reported to be influenced by the high internal pressure and accelerated increase in temperature associated with microwave-assisted extraction [42]. The CHP appears to have a hard (Fig. 2a) and wrinkled (Fig. 2b) surface, very similar to the surface morphology of the pectin sample analyzed previously by Refs. [11,43]. Along similar lines [44], reported similar surface morphology of extracted pectin using microwave-assisted extraction. Observed cavities in the CHP are attributed to the sharp intracellular temperature and rise in pressure associated with microwave-assisted extraction [45]. Raw material type and extraction mode largely determine the surface morphology of the extracted pectin. From the obtained results, it can be concluded that the surface morphology of CHP is influenced by microwave-assisted extraction. Commercial pectin was used for standard reference. SEM images for commercial pectin (Fig. 3a: commercial pectin at 5 KX and 2 μm; Fig. 3c: commercial pectin at10 KX and 2 μm) and CHP (Fig. 3b: CHP at 5 KX and 2 μm; Fig. 3d: CHP at 10 KX and and 2 μm) at same resolution and magnification are provided for the reference.

The major chemical and functional groups in the CHP were evaluated *via* FT-IR analysis. Fig. 4 illustrates the FT-IR spectra of the

Table 1
Purity of coffee husk pectin (CHP).

Molecular properties of Coffee Husk Pectin (CHP)	
Moisture (%)	1.52 ± 0.30
Ash (%)	2.03 ± 0.04
Equivalent weight (g/mol)	789.35 ± 8.47
Methoxyl level (%)	11.78 ± 0.26
Total anhydrouronic acid (AUA, %)	91.51 ± 1.98
Degree of esterification (DE, %)	73.08 ± 1.53

CHP and the standard pectin ranging from 500 to 4000 cm^{-1}. The spectra between 950 and 1300 cm^{-1} correspond to the fingerprint region for carbohydrates, explicitly for sugar composition [46]; Baum et al., 2016). This in turn further allows the identification of major chemical groups that are particular for specific polysaccharides [47]. The peak at 1750 cm^{-1} in the CHP represents the esterified carboxyl group, arising due to the ester carbonyl-stretching band [15]. The spectra region between 1500 and 1800 cm^{-1} corresponds to the degree of methylation [1]. Thus, the strong evolving peak at 1745 cm^{-1} in the CHP corresponds to a higher DE value. Also, obtained FT-IR spectrum for CHP is consistent with the experimentally determined % DE by titration method. Peaks around 1100 cm^{-1} in both CHP and standard pectin represent the pectin backbone [48]. The peak bending around 3400 cm^{-1} is reported due to the stretching of the hydroxyl groups [49]. This study is also in agreement with the results obtained from Hasanah et al. (2018) and Reichembach, and de Oliveira Petkowicz (2020) which yielded high methoxyl pectin (HMP) from the coffee bean.

XRD was analyzed to achieve more information about CHP structure (crystalline and amorphous). Fig. 5 depicts the XRD patterns of the CHP and standard pectin. The 2Θ values at 14.14°, 18.78°, 20.00°, 30.94°, 34.08° and 37.54° are reported for standard pectin [50]. The 2Θ values at 18.78° and 21.01° were observed for CHP, which are generally observed for pectin [51]. The XRD pattern for the CHP showed the crystallinity due to a typical diffractogramat 21.01° (2Θ). Similar results were observed by Ref. [52].

3.3. Molecular properties

The quality of the extracted CHP is ascertained by observing its purity. The purity of the CHP can be assessed by estimating its moisture and ash content, equivalent weight, methoxyl level, total anhydrouronic acid (AUA) %, and degree of esterification. The result of the molecular properties of the CHP is shown in Table 1.

Moisture in CHP was found to be 1.52 ± 0.30%. It indicates the amount of water present in the product which can be used by the microorganismsfor their metabolic activity [46]. Low moisture promotes better shelf life and prolongs the storage period by inhibiting the growth of microorganisms and the activities of hydrolytic enzymes (pectinases) that adversely affect pectin quality [53]. The ash content of CHP was found to be 2.03 ± 0.04%. This value is in a similar range as obtained for commercial pectin derived from apple pomace (1.96%) and citrus peels (3.46%) [46]. Ash levels represent pectin purity. The lower the ash content, the higher the purity of the pectin. The equivalent weight of pectin depends on the type of plant, quality of raw material, method of extraction, extraction solvent, and extraction process. The equivalent weight of CHP extracted using citric acid was found to be 789.35 ± 8.47 g/mol. This value is found to be considerably higher than the pectin extracted from citrus peel powder using citric (294.11 g/mol) and nitric (515 g/mol) acid as a solvent [24]. The pectin extracted from cocoa husk was found to be in the range of 510.68–645.19 g/mol [54] which is lower than that of CHP reported in this study. Lower equivalent weight represents higher partially degraded pectin and higher equivalent weight pectin exhibits a greater degree of gel-forming ability [24]. Methoxyl content represents the total moles of methyl alcohol in 100 mol of galactouronic acid and this strongly determines the ability of the pectin to form gel [54]. Methoxyl content of CHP was estimated to have 11.78 ± 0.26%. This value is higher than the methoxyl content (6.98%) of commercial pectin and the pectin obtained from Robusta coffee pulp (2.21%) [55]. Thereof, these values strongly support the fact that methoxyl levels vary with the source of material and the extraction method. Based on the methoxyl content in this study, CHP can be categorized as HMP (Methoxyl content >7%) [56]. The AUA of CHP was found to be 91.51 ± 1.98%. AUA content indicates the pectin purity and a value less than 65% is found unsuitable for food and pharmaceutical applications [46]. The low value of AUA means that the extracted pectin might not be sufficiently pure with the possible presence of protein, starch, and sugars in the precipitated pectin [57]. The AUA % in the present study indicates that the CHP is of high purity and can be used for food and pharmaceutical applications. The DE of CHP was found to be 73.08 ± 1.53% and this value is higher than that of commercial pectin (50.84%). The result of our study is in agreement with the pectin extracted from Arabica coffee pulp [2]. The lower DE value is attributed to the conversion of pectin into protopectin [24]. The DE % in the present study indicates that the CHP is of high ester pectin (DE > 50%), which has the ability to form rapid gels [58]. The use of a citric acid solution to extract pectin results in the pectin of higher DE %. This positive effect of citric acid is also validated by other researchers [59,60].

3.4. Evaluation of functional properties

3.4.1. Antioxidant activity of CHP

Exposure to xenobiotic compounds from various foods and environment causes significant production of free radicals *in vivo* [34]. They are reported to cause cellular oxidative damage that may be related to the onset of many disorders [2]. Evaluating the antioxidant ability of CHP paves way for its biomedical application. The antioxidant activity of CHP was evaluated by DPPH, FRAC, ABTS free

Table 2
IC_{50} values of ascorbic acid and Coffee Husk Pectin (CHP).

Ascorbic acid			Coffee Husk Pectin (CHP)		
Concentration (μg/mL)	% DPPH scavenging	IC_{50} (μg/mL)	Concentration (mg/mL)	% DPPH scavenging	IC_{50} (mg/mL)
5	5.73	15.45 ±	0.5	11.83	4.87 ± 0.29
7.5	10.65	0.02	1	23.07	
10	21.31		3	33.72	
12.5	39.34		5	51.47	
15	48.36		10	74.55	

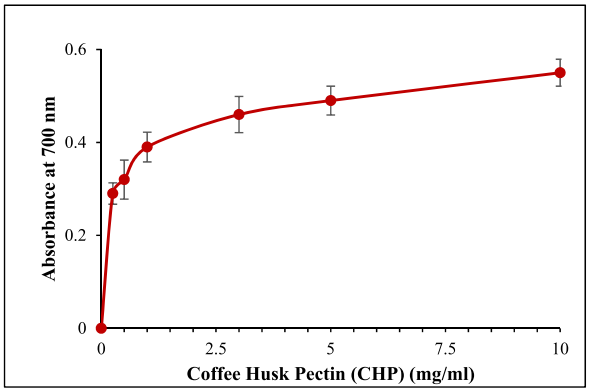

Fig. 6. Ferrous reducing antioxidant capacity (FRAC) assay of Coffee Husk Pectin (CHP).

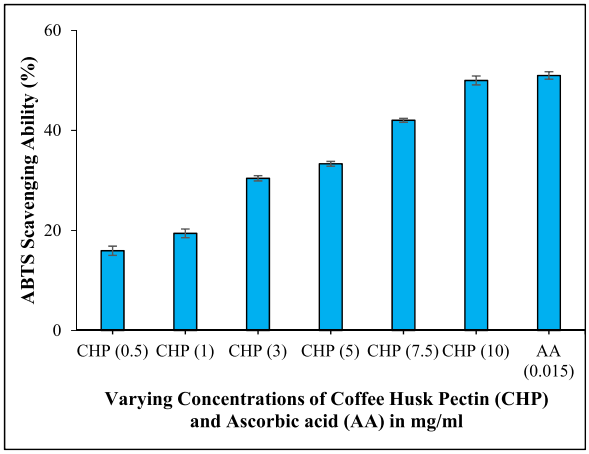

Fig. 7. ABTS Scavenging ability of Coffee Husk Pectin (CHP). **All values are expressed as Mean ± SD (n = 3). No significance was observed between CHP (10) versus AA (0.015)**.

radical scavenging, and ascorbate auto-oxidation assays. CHP exhibited significant DPPH scavenging, reducing ability, ABTS free radical scavenging, and ascorbicacid auto-oxidation inhibition effects. DPPH is an organic radical widely used in evaluating the antioxidant potential of various natural compounds. The DPPH scavenging ability can be evaluated by determining the IC_{50} value, which corresponds to the concentration of CHP required to scavenge 50% of the DPPH radical resent in the reaction mixture. The scavenging ability of CHP was compared to ascorbic acid. DPPH scavenging ability increased with an increase in CHP concentration. The ascorbic acid demonstrated the IC_{50} value of 15.45 ± 0.02 μg/mL and 4.87 ± 0.29 mg/mL was obtained for CHP (Table 2). A recent study by Ref. [14] reported a similar IC_{50} value of 4.31 mg/mL for the pectin extracted from coffee husk. Our results are also in line with the DPPH scavenging ability reported earlier by Wikiera et al. (2021) for commercial pectin (5.24 ± 0.08 mg/mL) and acid extracted pectin (9.01 ± 0.11 mg/mL). Furthermore [61,62], reported the IC_{50} value for DPPH scavenging assay for the pectin from fig skin (*Ficuscarica* L.) and apple pomace as ~6.8 mg/mL and >4.6 mg/mL, respectively. This confirms the DPPH scavenging ability of CHP.

The ability of CHP to reduce the Fe^{3+} - ferricyanide complex to the ferrous form by donating an electron is evaluated by FRAC assay.

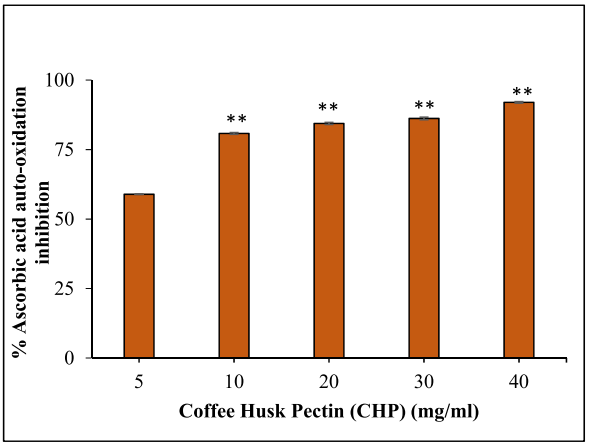

Fig. 8. Ascorbic acid auto-oxidation inhibition by Coffee Husk Pectin (CHP). **All values are expressed as Mean ± SD (n = 3). Significance difference (**$P < 0.01$) was observed between CHP (5 mg/mL) versus CHP (10, 20, 30 & 40 mg/mL).**

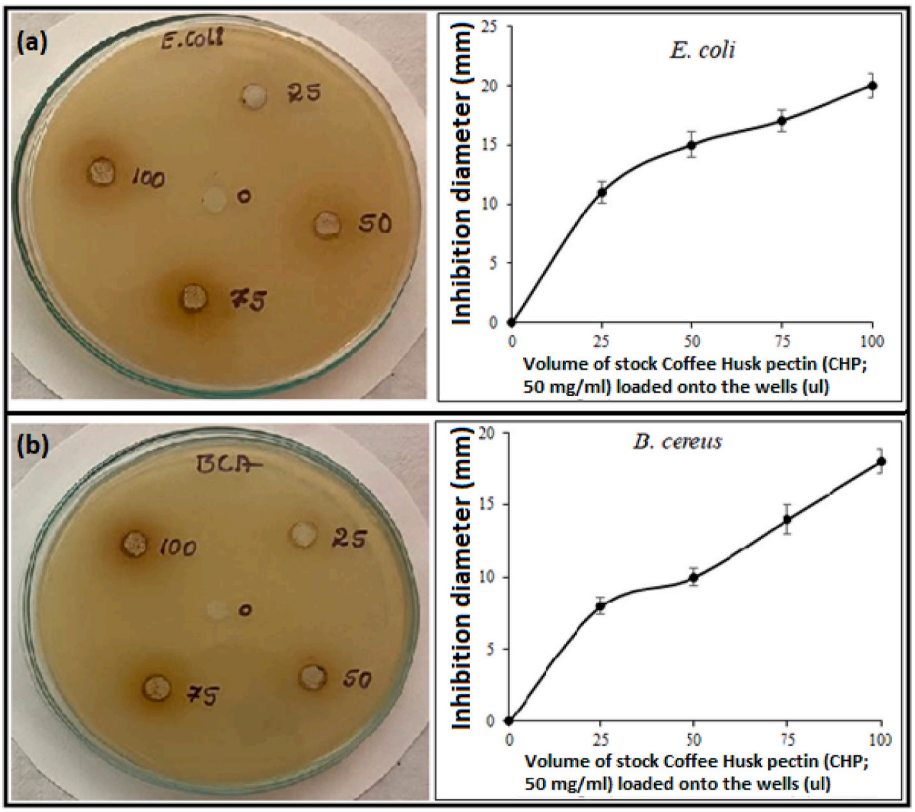

Fig. 9. Antibacterial activity of Coffee Husk Pectin (CHP) against *E. coli* (a) and *B. cereus* (b).

The FRAC of CHP increased with an increase in its concentration as evident with an increase in absorbance at 700 nm (Fig. 6). CHP concentration of 5 ± 0.02 mg/mL was required to produce 0.5 absorbance unit at 700 nm.

Further to confirm the antioxidant activity of CHP, the ABTS radical scavenging assay was also performed. This assay is often used to determine the total antioxidant activity of natural biomolecules [63]. The concentration-dependent ABTS radical scavenging activity of CHP is presented in Fig. 7. No significant difference in the ABTS scavenging activity was observed at the highest pectin concentration (10 mg/mL) tested and at the concentration of standard ascorbic acid evaluated (15 μg/mL). The IC_{50} value of CHP for ABTS radical scavenging activity was found to be 8.69 ± 0.14 mg/mL [64]. reported the IC_{50} value of acid-soluble pectin (crude) and acid-soluble pectin for ABTS radical scavenging activity as 7.1 and 4.8 mg/mL, respectively. In our study, CHP exhibited superior

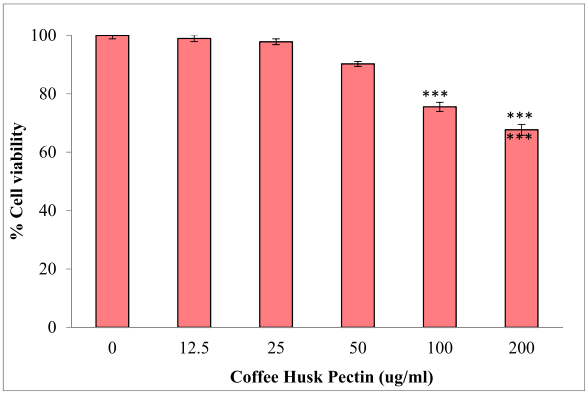

Fig. 10. Effect of Coffee Husk Pectin (CHP) on cell viability of RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were incubated with and without indicated concentrations of coffee husk pectin for 24 h. Data represents mean ± SD, n = 3. Significanty difference (***P < 0.001) was observed between untreated versus CHP (100 & 200 mg/mL).

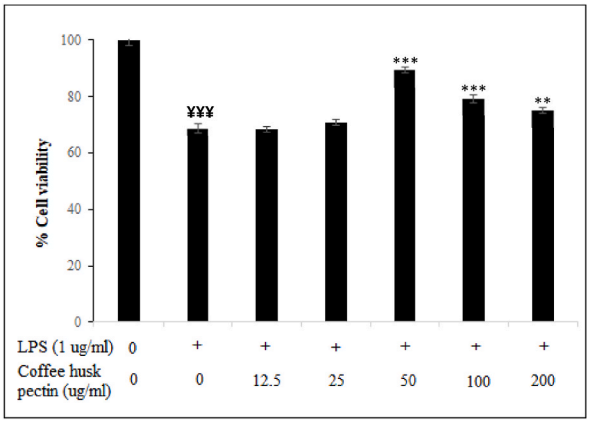

Fig. 11. Inhibition of LPS-induced cytotoxicity on RAW 264.7 cells by Coffee Husk Pectin (CHP). Cells (2×10^4 cells/well) in 96-well plates were first incubated with LPS (1 μg/mL) for 4 h, followed by 24 h of incubation with varying concentration of coffee husk pectin. Data represents mean ± SD, n = 3. Significant difference (***P < 0.001) was observed between untreated versus LPS-CHP treated; ¥¥¥P < 0.001 between untreated versus LPS treated.

DPPH scavenging ability (4.87 ± 0.29 mg/mL) than ABTS radical scavenging activity (8.69 ± 0.14 mg/mL). However, the antioxidant ability of food materials based on DPPH and ABTS is reported to be inconsistent [63]. The antioxidant ability of pectin is attributed to the higher number of carboxyl and hydroxyl groups in its backbone, conferring as electron donors to free radicals [17] and for its ability to terminate free radical chain oxidation reaction to form a stable compound by combining the radical ions [65].

Ascorbic acid is an essential nutrient reported to play a crucial role in redox, metabolic and epigenetic pathways [66]. Humans fail to produce endogenous ascorbic acid and thus, it is obtained exogenously through food. It is the major antioxidant found in the lungs (scavenges reactive oxygen species (ROS) [14], obtained from their surroundings following intestinal absorption and blood circulation through sodium-dependent vitamin C transporters [22]. The antioxidant properties of ascorbic acid in the lungs are considered extremely beneficial for chronic obstructive pulmonary disease (COPD), helping to reduce lung damage [66]. Ascorbic acid availability is diminished extremely through the destruction by its auto-oxidation [67]. This strongly accounts for its loss of biological activity [68]. CHP was found to inhibit ascorbic acid auto-oxidation in a dose-dependent manner (Fig. 8). CHP (40 mg/mL) exhibited 88.01 ± 0.28% of the ascorbate auto-oxidation inhibition *in-vitro*.

3.4.2. Antibacterial activity

Pectin from different sources is reported to offer antibacterial activity against gram-positive and gram-negative bacteria [21,29, 69]. The antibacterial activity of CHP was assessed at various concentrations against *E. coli* and *B. cereus* (Fig. 9). The zone of inhibition was in the range of 8–20 mm for both strains. The highest volume of CHP (corresponding concentration 5 mg/mL) tested demonstrated maximum inhibition diameter for *E. coli* (20 ± 1.01 mm; Fig. 9a) and *B. cereus* (18 ± 0.84 mm; Fig. 9b). The results are in line with [3] and Chaiwarit et al. (2018) wherein edible pectin film (*E. coli* and *B. cereus*) and pectin from mango peel (*S. aureus*) demonstrated antibacterial activity. All these data indicate that pectin from various sources can be used as an antibacterial compound and possess

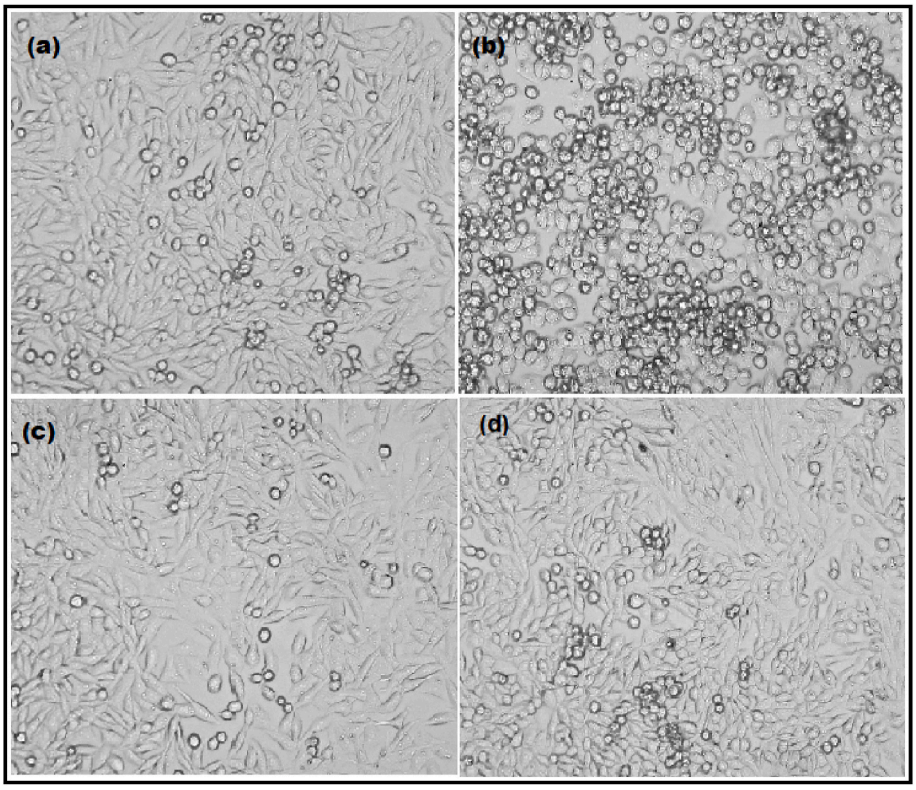

Fig. 12. RAW264.7 Cell Morphology. (a) Untreated RAW264.7 cells (b) RAW264.7 cells exposed to 1 μg/mL Lipopolysaccharides (LPS) (c) RAW264.7 cells exposed to 50 μg/mL Coffee Husk Pectin (CHP) (d) RAW264.7 cells exposed to 1 μg/mL Lipopolysaccharides (LPS) and 50 μg/mL Coffee Husk Pectin (CHP).

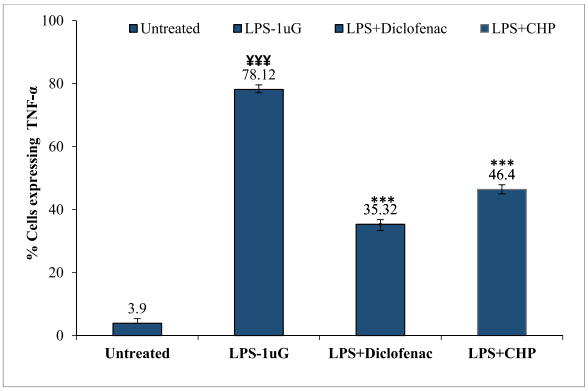

Fig. 13. Percentage of RAW264.7 cells expressing TNF-α. **Data represents mean ± SD, n = 3. Data were analyzed by one-way ANOVA followed by a post-hoc Dunnett test. ¥¥¥*P < 0.001*, Untreated versus LPS treated. ****P < 0.001*, LPS treated versus LPS + CHP treated and LPS + Diclofenac treated.**

potential in the development of edible film packages [21].

3.4.3. In-vitro cytotoxicity using MTT assay

The beneficial roles of pectin from various sources under the conditions of inflammation have been previously reported [70,64]. To set the experimental conditions for evaluating the anti-inflammatory potential of CHP, the cytotoxicity was assessed by treating varying concentrations of CHP (12.5, 25, 50, 100, and 200 μg/mL) against RAW264.7 macrophages (Fig. 10). **The monocyte/macrophage-like cell line RAW 264.7, originating from Abelson leukemia virus transformed cell line derived from BALB/c mice, has been one of the most widely used cell line for evaluating anti-inflammatory property** [71]**. The cell lines**

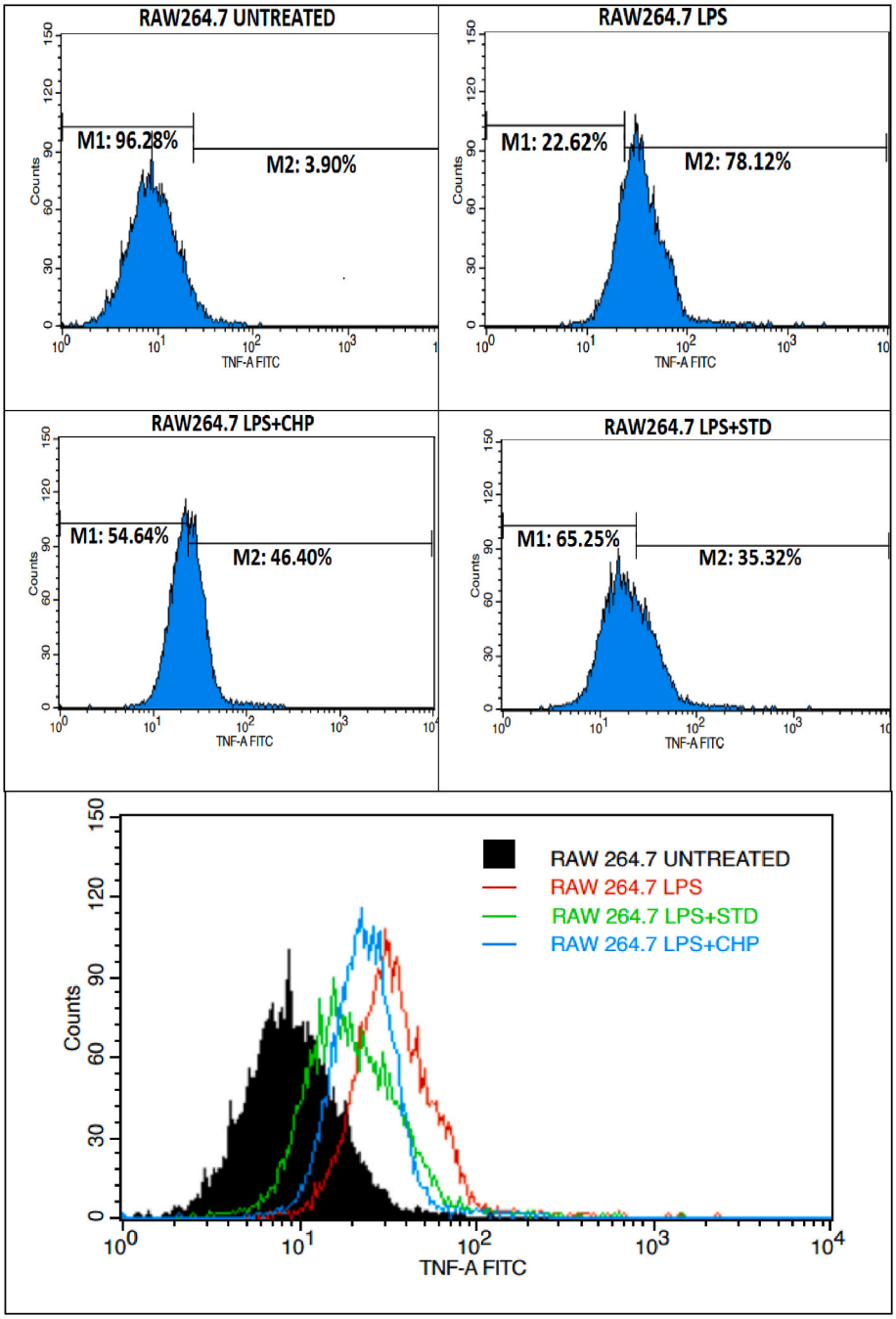

Fig. 14. Effect of Coffee Husk Pectin (CHP) on TNF-α expression against LPS induced RAW264.7 cells. TNF-α histogram of the gated RAW264.7 singlets distinguishes cells at the M1 and M2 phases. (Here M1 refers to negative expression/region and M2 refers to the Positive expression/region). Gating of M1 and M2 regions is approximate and can be refined using Cell Quest Pro Software, Version 6.0 software. % of cells observed in M2 region is considered as TGF- β expression in the present study.

were obtained from healthy mouse model. Significant number of scientific evidences demonstrate the use of RAW 264.7 cell lines to evaluate the immune-modulatory activities of natural and/or synthetic compounds [72,73,74]**. Safety of the coffee husk pectin is also a question while evaluating its immunomodulatory effect against LPS infected host macrophage cells. From this view, cytotoxic effect of CHP was also evaluated on the same lines** [4]

The CHP showed cell viability >75% at the concentration of 100 μg/mL and below, which indicates that CHP offered poor cytotoxic effect against RAW264.7 macrophages at concentrations above 100 μg/mL. However, the cell viability was found to be 67.65 ± 1.87% for CHP (**$p < 0.001$) at 200 μg/mL. These data indicate that CHP does not affect RAW264.7 cells viability at the concentrations of

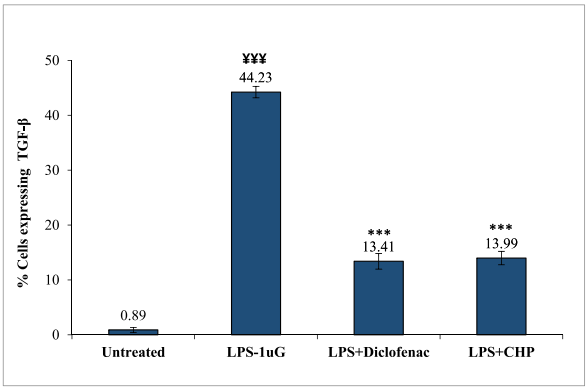

Fig. 15. Percentage of RAW264.7 cells expressing TGF-β. **Data represents mean ± SD, n = 3. Data were analyzed by one-way ANOVA followed by a post-hoc Dunnett test. ¥¥¥*P < 0.001*, Untreated versus LPS treated. ****P < 0.001*, LPS treated versus LPS + CHP treated and LPS + Diclofenac treated.**

0–50 μg/mL and provides good biocompatibility.

Exposure of RAW264.7 cells to LPS (1 μg/mL), caused a significant (***p < 0.001*) decline in cell viability (viability reduced by 31.47% in comparison to control) (Fig. 11). Exposure of CHP (12.5–200 μg/mL) to LPS treated RAW264.7 cells, caused alterations in cell viability. While a significant increase (***p < 0.001*; **p < 0.01*) in cell viability was observed between LPS-treated and CHP (≥50 μg/mL) exposed LPS-treated RAW264.7 cells, no significant increase in cell viability was observed between the LPS treated and all other CHP (<50 μg/mL) exposed samples. These results are in line with the pectin extracted from okra *(Abelmoschusesculentus* L.*)* [64].

The morphology of RAW264.7 cells can be seen in Fig. 12. The figure shows the morphology of RAW264.7 cells before and after treatment with LPS and CHP. Fig. 12a shows the morphology of untreated RAW264.7 cells which are both loosely adherent spindle-shaped cells and rounded viable cells [75]. Exposure to RAW264.7 cells to LPS changed its morphology into an irregular form with pseudopodia formation (Fig. 12b) [76]. This irregular cell morphology with pseudopodia formation was found to be significantly lesser in the RAW264.7 cells treated with CHP (Fig. 12c) and in the LPS-exposed RAW264.7 cells treated with CHP (Fig. 12d). These data suggest the ability of CHP to prevent damage caused by LPS as evident from cell morphology.

3.4.4. Anti-inflammatory activity using flow cytometry

The bacterial endotoxin LPS, present in the outer membrane of Gram-negative bacteria is detected in the IBD patient's plasma [34]. Activation of macrophages either by enteric bacteria and/or their constituents *viz.,* LPS induces significant alterations in the levels of pro-inflammatory cytokines and chemokines in the bloodstream [77,78]. Exaggerated inflammatory response mediated by exogenous LPS compounds might trigger severe inflammation in the human body [79]. In the present study, the anti-inflammatory activity of CHP on LPS-induced TNF-α and TGF-β levels by RAW264.7 cells was evaluated. As shown in Fig. 13, a significant increase in the percentage of cells expressing TNF-α was observed after exposure to LPS (78.12 ± 1.04%) (***p < 0.001*), whereas treatment with CHP, caused a sustained decrease in LPS-induced elevation in TNF-α levels (46.40 ± 1.45%) (***p < 0.001*). The effect of CHP in decreasing cells expressing TNF-α was similar to that of a standard drug (Diclofenac) (35.32 ± 1.98%). Fig. 14 shows the representative FACS histograms where M1 corresponds to the region of cell percentage not expressing TNF-α and M2 corresponds to the region of cell percentage expressing TNF-α. Exposure of RAW264.7 cells to LPS caused a significant increase in the cells expressing TNF-α (78.12%) in comparison to the control (3.90%) as evident from the histograms. An increase in cell population expressing TNF-α level induced by LPS was significantly inhibited upon exposure to CHP (46.40%) and Diclofenac (35.32%). However, no significant difference between CHP and Diclofenac-treated cells was observed.

Similar observations were reported for TGF-β (Figs. 15 and 16). LPS induced elevation in the cells expressing TGF-β (44.23 ± 1.05%) (***p < 0.001*) were significantly reduced by CHP (13.41 ± 1.34%) (***p < 0.001*) and Diclofenac (13.99 ± 1.23%) (***p < 0.001*) (Fig. 15). Recent studies have also documented the *in vivo* anti-inflammatory potential of pectin from orange and citrus peels using 2,4,6-trinitrobenzoic sulfonic acid (TNBS) induced colitis mice models. Their results demonstrated the ability of orange pectin to attenuate experimentally induced colitis by modulating pro-inflammatory cytokine (IL-6) levels [80]. Pectin from functional food, *Rubuschingii* Hu also demonstrated anti-inflammatory activity by suppressing the levels of TNF-α [70]. To the author's knowledge, this is the first communication exploring the antibacterial and anti-inflammatory potential of CHP.

4. Conclusion

A huge amount of coffee husk generated during coffee bean processing contains valuable bioactive compounds which can be of commercial interest. Biotechnological approaches are of the current day need to valorize coffee husk to avoid environmental problems. This study describes the use of coffee husk as a source of biologically active pectin. In the present study, coffee husk was used a source to extract pectin using microwave assisted extraction technique. The extracted CHP was characterized and its biological activity was evaluated. CHP exhibited high antioxidant potential with low IC_{50} value for DPPP free radical scavenging assay. Elevated levels of

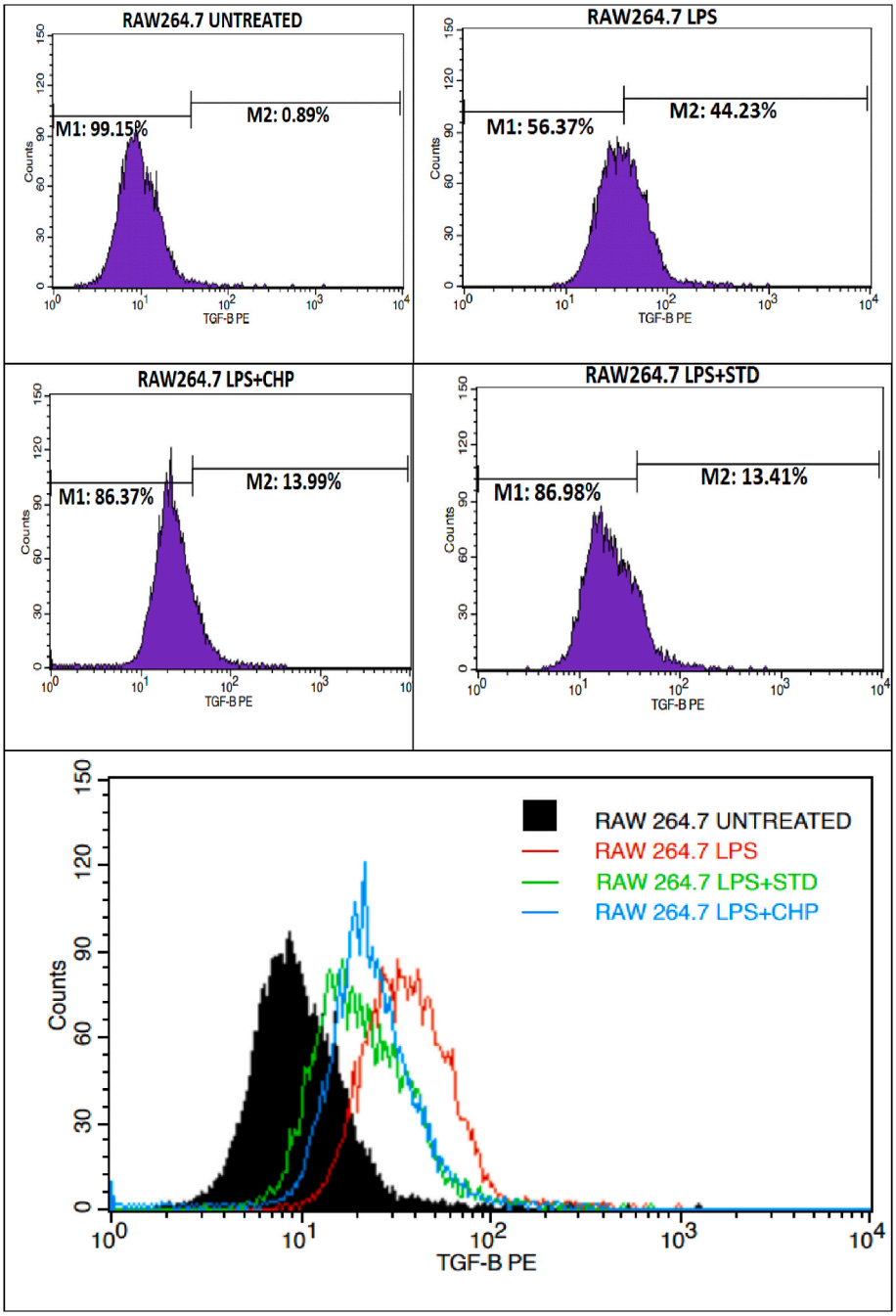

Fig. 16. Effect of Coffee Husk Pectin (CHP) on TGF-β expression against LPS induced RAW264.7 cells. TGF- β histogram of the gated RAW264.7 singlets distinguishes cells at the M1 and M2 regions. (Here M1 refers to negative expression/region and M2 refers to the positive expression/region). Gating of M1 and M2 regions is approximate and can be refined using Cell Quest Pro Software, Version 6.0 software. % cells observed in M2 region is considered as TGF-β expression in the present study.

inflammatory markers *viz.,* TNF-α and TGF-β produced by activated immune cells may pave way for pathogenesis of chronic diseases. CHP offered anti-inflammatory activity by controlling the production of inflammatory mediators. Thus, CHP can be viewed as an extremely attractive therapeutic molecule. Taken together, all these results suggest that coffee husk can be exploited commercially as a source of pectin and pectin from coffee husk can be used for food and non-food applications. Our future studies are directed towards understanding the mechanism of CHP in mitigating LPS-induced macrophage inflammation.

Author contribution statement

G Divyashri: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

T P Krishna Murthy; K Vasanth Ragavan; Anjanapura Venkataramanaiah Raghu: Contributed reagents, materials, analysis tools or data.

GM Sumukh; Lingam S Sudha; S Nishka; Gupta Himanshi; N Misriya; B Sharada: Performed the experiments.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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