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Unlocking the nutraceutical potential of polysaccharides from snake grass (*Clinacanthus nutans*)

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ABSTRACT

The therapeutic potential of Sabah snake grass (*Clinacanthus nutans*) is ascribed to its phytochemical compounds yet the leaf polysaccharides, which are present in the traditional herbal drink, remain underexplored. This study aimed to investigate and characterize the bioactive polysaccharide of *C. nutans* leaves via acid-based buffer extraction and evaluate their *in vitro* multifunctional bioactivities for potential nutraceutical applications that require further validation. Acid-based extraction of *C. nutans* leaves yielded 11.2 ± 1.0% polysaccharide with antioxidant, anti-hypertensive and anti-diabetic effects. For instance, 1 mg/ml of *C. nutans* polysaccharide gave 37.12 ± 0.8 mM ferric reducing antioxidant power (FRAP), 60.8 ± 6.9% angiotensin I-converting enzyme (ACE) inhibitory activity and 55.3 ± 8.1% α-glucosidase inhibitory activity. The bioactivities of *C. nutans* polysaccharide were ascribed to its mild acidic property (pH 4.30 ± 0.04) or phenolic content (1.9 ± 0.5%). Moreover, Fourier-transformed infrared (FTIR) analysis suggested the *C. nutans* polysaccharide as a pectic-polysaccharide with a small branch size and low degree of esterification (14%). The functional groups in the pectic-polysaccharide such as hydroxyl and carboxyl groups were postulated to interact with Fe^{3+} ion, ACE enzyme and α-glucosidase enzyme for the bioactivities. Overall, this study explored the physicochemical profile, and the current results showed potential health benefits of *C. nutans* polysaccharide.

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1. INTRODUCTION

Clinacanthus nutans (Burm.f) Lindau is one of the important species from Acanthaceae family found throughout Southeast Asia. Traditionally, this medicinal herb was used to treat skin rashes, insect bites, snakebites, lesions caused by herpes diabetes, diarrhoea, cancers and gout (Alam et al., 2016; Aliyu et al., 2020). This plant also exhibits anti-inflammatory, anti-microbial, anti-viral, anti-hyperlipidemia, vasorelaxation, and renoprotective activities (Nik Abd Rahman et al., 2019; Ong et al., 2022). These pharmacological activities could be attributed to its bioactive compounds, including saponins, glycosides, steroids, terpenoids, and flavonoids (Aliyu et al., 2020). The plant leaf was the most used part compared to roots, stem and whole parts of *C. nutans*. It is prepared by either decocting the leaves with water for oral ingestion or immersing in alcohol for topical application.

Polysaccharides, which are complex carbohydrates extracted from leaves, represent an underexplored bioactive component of *C. nutans*. Plant polysaccharides are non-toxic, resistant to human digestion and absorption that exert immune-enhancing functions without being absorbed into the bloodstream (Kumar et al., 2020; Chen et al., 2021). The diverse compositions and structures of plant polysaccharides contribute to their functional properties and potential therapeutic benefits, including immunoregulatory, anti-inflammatory, hypoglycemic, antibacterial, antioxidant and antitumor activities (Minzanova et al., 2018; Ullah et al., 2019). In recent years, interest has expanded significantly toward the extraction, purification, and multifunctional characterization of leaf-derived heteropolysaccharides, demonstrating their high value as functional food ingredients and natural value-added matrixes (Mo et al., 2023; Chen et al., 2024; Nie et al., 2024). Despite extensive reports on the health benefits of *C. nutans* phytochemicals (Khoo et al., 2018), to our knowledge, no comprehensive characterization of *C. nutans* leaf polysaccharides and their multifunctional activities has been reported, which represents a novel area of exploration. Therefore, this study addresses this research gap by evaluating the antioxidant, anti-hypertensive and anti-diabetic activities of *C. nutans* polysaccharides. This is followed by physicochemical characterization and its correlation with bioactivities. These findings are anticipated to contribute to the pharmaceutical and nutraceutical industries by introducing new natural value-added components.

2. MATERIAL & METHODOLOGY

2.1 Plant materials and chemicals

C. nutans leaves were retrieved from Taiping, Perak, Malaysia and authenticated by the Unit of Herbarium, School of Biological Sciences, Universiti Sains Malaysia (specimen voucher: 11153). The collected leaves were rinsed, lyophilized, milled, sieved (30 mesh) and stored at 4 ℃ prior to extraction. Chemicals used in this study were sourced from Sigma-Aldrich (Malaysia) unless stated otherwise.

2.2 Extraction of polysaccharide from *C. nutans* leaves

C. nutans polysaccharide was extracted as described previously (Alba et al., 2015) with modifications, where a 0.04 mg/mL sample was prepared using 0.1 M citrate-phosphate buffer (pH 2) and constantly shaken (250 rpm, 120 min, 80 ℃). The slurries were filtered (pore size 50-75 μm) and centrifuged at 5000 rpm for 20 min at 20 ℃ to remove insoluble debris and obtain the clear supernatant. The polysaccharides were added to four volumes of 99% ethanol in a 4 ℃ chiller overnight. Precipitates were collected by

centrifugation, lyophilized and stored in a desiccator at room temperature until further analysis. Extraction yield of *C. nutans* polysaccharide was calculated as:

$$\% \text{ Yield (w/w)} = \left(\frac{Wp}{Wo}\right) \times 100 \quad (1)$$

where Wp and Wo are the weights of lyophilized polysaccharide and initial sample (dry basis), respectively.

2.3 Bioactivity assays

The bioactivity assays were conducted using 1 mg/mL *C. nutans* polysaccharide solution unless specified. This concentration was selected as a uniform screening benchmark optimized for the preliminary evaluation of crude plant-derived heteropolysaccharides. Utilizing a baseline of 1 mg/mL facilitates direct comparative profiling against historical values for equivalent structural matrixes prior to expanding into target-specific, dose-dependent downstream validation (Ma et al., 2018; Soua et al., 2020; Jiang et al., 2024).

2.3.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

For DPPH assay, the polysaccharide was added to 0.1 mM methanolic DPPH solution at 1:30 (v/v) ratio (Liu et al., 2009). The mixture was protected from light and incubated at 30 ℃ for half an hour. Absorbance was measured at 517 nm (Spectramax M5, Molecular Devices, US). The radical scavenging ($\%\text{DPPH}_{\text{sc}}$) was calculated as:

$$\%\text{DPPH}_{\text{sc}} = \left(A_{con} - A_{sample}\right) \times 100 / A_{cont} \quad (2)$$

where A_{con} and A_{sample} represent the absorbance of the control and sample, respectively.

2.3.2 Ferric reducing antioxidant power (FRAP)

FRAP assay was performed based on a modified method (Benzie & Strain, 1999). The FRAP reagent contained 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 0.3 M acetate buffer at pH 3.6 at 1:1:10 ratio. This freshly prepared FRAP reagent (600 µL) was then mixed with 40 µL *C. nutans* polysaccharide. The mixture was incubated at 37 ℃ for 60 min, and the absorbance was monitored at 593 nm. Results were compared against $FeSO_4 \cdot 7H_2O$ (0–2 mM) standards (Shui & Leong, 2006) and expressed in mM $FeSO_4$.

2.3.3 ACE inhibition

ACE inhibition assay was carried out as previously described (Cushman & Cheung, 1971). Fifty µL *C. nutans* polysaccharide was added to 50 µL ACE (50 mU/mL) and incubated at 37 ℃ for 10 min. Subsequently, 150 µL N-Hippuryl-His-Leu (HHL; 4.15 mM in 50 mM borate buffer containing 0.3 M NaCl) was added and further incubated at 37 ℃ for 30 min. The enzymatic reaction was then stopped by adding 500 µL HCl (1 M), and hippuric acid was extracted by adding 1.5 mL ethyl acetate. After 5 min, the top layer was air-dried at 45 ℃. The pellet was redissolved in 1 mL ddH_2O and quantified at 228 nm using a quartz cuvette. Captopril was used as a positive control. ACE inhibition was calculated as:

$$\%ACE\ Inhibition = \left(A_{con} - A_{sample}/A_{cont}\right) \times 100 \quad (3)$$

where A_{con} and A_{sample} represent the absorbance of the control (without inhibitor) and sample, respectively.

2.3.4 ACE DPP4 (dipeptidyl peptidase-4) inhibition

The DPP4 inhibition assay was conducted as previously reported (Nongonierma & Fitzgerald, 2013), where the enzyme and substrates were dissolved in 100 mM Tris-HCl buffer (pH 8). Briefly, 25 µL *C. nutans* polysaccharide was mixed with 67 µL DPP4 (2.5 µU/mL, then incubated at 37 ℃ for 10 min. Subsequently, 67 µL GP-pNA (0.2 mM), was added and incubated at 37 ℃ for 1 h. The absorbance was measured at 405 nm. Diprotin A was used as a positive control. The inhibition of DPP4 was calculated using Eq. (4):

$$\%\ DPP4\ Inhibition = \left(A_{con} - A_{sample}/A_{cont}\right) \times 100 \quad (4)$$

where A_{con} and A_{sample} represent the absorbance of the control (without inhibitor) and sample, respectively.

2.3.5 α-Amylase inhibition

The ability of *C. nutans* polysaccharide to inhibit α-amylase was performed as previously described by Worthington (1993). Briefly, 500 µL sodium phosphate buffer (0.02 M, pH 6.9, in 6 mM NaCl) containing 0.5 mg/mL enzyme was added to 100 µL *C. nutans* polysaccharide. The mixture was left at 25 ℃ for 10 min before adding 500 µL of 1% starch. Another round of incubation followed, and then the reaction was terminated with 1 mL of dinitrosalicylic acid reagent. The sample was heated at 100 ℃ for 5 min, followed by cooling to room temperature. Sample was diluted with 10 mL ddH_2O and the absorbance was measured at 540 nm. The inhibition of α-amylase was calculated as:

$$\%\ amylase\ inhibition = \left[A_{con} - (A_{sample} - A_{blank})\right]/A_{con} \times 100 \quad (5)$$

where A_{con} and A_{sample} represent absorbances of control (without inhibitor) and sample, respectively. A_{blank} represents absorbance of starch and sample without enzyme.

2.3.6 α-Glucosidase (AG) inhibition

The AG inhibition assay was conducted as previously described by Kamal *et al.* (2018). Briefly, 10 mM 4-nitrophenyl-β-d-glucopyranoside (pNPG) solution was dissolved in 0.1 M phosphate buffer (pH 6.8) and then mixed with 80 µL *C. nutans* polysaccharide solution. The AG enzyme (20 µL, 1 U/mL) was added and incubated at 37 ℃ for 10 min. The production of p-nitrocophenol from pNPG was measured at 405 nm. The percent inhibition of AG was calculated as:

$$\%AG\ inhibition = \left[A_{con} - (A_{sample} - A_{blank})\right]/A_{con} \times 100 \quad (6)$$

where A_{con} and A_{sample} represent the absorbance of the control (without inhibitor) and sample, respectively. A_{blank} represents absorbance of pNPG and sample without enzyme.

2.4 Physicochemical characterization of *C. nutans* polysaccharide

2.4.1 pH

The pH of 1.0% (w/v) *C. nutans* polysaccharide was determined using a pH meter (Mettler Toledo, USA). Three replicates of pH readings were recorded.

2.4.2 Protein content

Protein contamination in *C. nutans* polysaccharide was evaluated by Bradford assay (1976). Briefly, polysaccharide was added to Bradford reagent (ratio 1:50 v/v) and left at room temperature for half an hour. Absorbance was then recorded at 595 nm. Protein content was calculated using bovine serum albumin (BSA) standard curve (0–1 mg/mL).

2.4.3 Total phenolic content (TPC)

A previously described protocol by Shui *et al.* (2006) was used to detect the amount of phenolics in *C. nutans* polysaccharide. Briefly, 40 µL polysaccharide (1 mg/mL) was mixed with 1.8 mL Folin-Ciocalteu (prediluted 10×) and left at room temperature for 5 min. Sodium bicarbonate (1.2 mL, 7.5% (w/v)) was added and incubated under a dark setting for 1 h. Absorbance was measured at 765 nm. Gallic acid (0–1 mg/mL) was prepared as the standard. The result was expressed as gallic acid equivalent (GAE)/mg *C. nutans* polysaccharide.

2.4.4 Total carbohydrate content

The carbohydrate content of *C. nutans* polysaccharide was evaluated through phenol-sulfuric method (Dubois et al., 1956). Briefly, 75 µL of polysaccharide (0.1 mg/mL) was mixed with 75 µL of 5% (w/v) phenol and placed on an ice bed before adding 375 µL of concentrated H_2SO_4. The mixtures were incubated at 80 ℃ for 30 min. Absorbance was measured at 495 nm. The carbohydrate content was calculated against a maltose standard curve (0–18 mg/mL).

2.4.5 Monosaccharide composition

The sugar monomers and their compositions in *C. nutans* polysaccharide were determined using high-performance liquid chromatography (HPLC) according to Lv et al. (2009). Briefly, 1 mg/mL polysaccharide was degraded using 3 M trifluoroacetic acid (95 ℃, 8 h). The sample was centrifuged (4000 rpm, 5 min), and the supernatant was vacuum dried before dissolving in 1 mL ddH_2O. Subsequently, 200 µL of methanolic 1-phenyl-3-methyl-5-pyrazolone (0.5 M) and 300 µL NaOH (0.3 M) were added to 100 µL of the sample. The mixture was heated at 70 ℃ for 1 h and cooled before adding 300 µL HCl (0.3 M). The sample was extracted with 1 mL of chloroform. The top layer was filtered (pore size 0.45 µm) and analysed (1260 Infinity Agilent Technology, USA). The monosaccharide compounds were separated using Zorbax SB-C18 reversed-phase column (250 mm×4.6 mm, Ø 5 µm, Agilent Technology).

2.4.6 Fourier-transformed infrared (FTIR) analysis

The polysaccharide functional groups were determined using FTIR spectrometer with an attenuated total reflectance (ATR) system (Cary 600 Series, Agilent Technologies, USA). The scan spectrum was recorded at 4000–650 cm^{-1} at 4 cm^{-1} resolution with 32 scans, scan speed 5 kHz, and sensitivity of 1. The resulting spectra were analyzed using Resolution Pro version 5.2.0 software.

2.5 Statistical analysis

All the experiments were conducted in three replicates and expressed as the average value ± standard deviation. Analysis of variance (ANOVA), followed by Duncan's test at 95% confidence level was conducted for statistical analysis using IBM SPSS Statistics 29 software.

3. RESULTS & DISCUSSION

3.1 Extraction yield of *C. nutans* polysaccharide

Conventional acid extraction of polysaccharides often results in high yields (>10%) of polysaccharides through cleavage of polysaccharide-protein linkages at the cell wall. In this study, the extracted *C. nutans* polysaccharide appeared as white powder, which gave a yield of 11.2 ± 1.0%. Comparison of yield with other leaf species revealed the influence of extraction methods and mechanisms, such as a lower yield of *Capparis spinosa* L. polysaccharide via hot water extraction (Mazarei et al., 2017) and a higher yield of *Suaeda fruticosa* (L.) Forssk polysaccharide via ultrasonic-assisted extraction (Mzoughi et al., 2018). This suggests further optimization of *C. nutans* polysaccharide extraction method for yield maximization.

3.2 *In vitro* bioactivities of *C. nutans* polysaccharide

3.2.1 Antioxidant capacity

The extracted *C. nutans* polysaccharide exhibited poor DPPH scavenging activity (9.3%) but a FRAP value of 0.11 mM FeSO4 at 1 mg/mL (Table 1). In comparison, the reference phenolic antioxidant gallic acid (1 mg/mL) demonstrated a markedly higher DPPH scavenging activity of 81.67% and a FRAP value of 16.6 mM $FeSO_4$. This means that while the crude leaf polysaccharide possesses measurable electron-donating metal-reducing capacity, its radical scavenging kinetics remain lower than pure, low-molecular weight phenolic standards. Several literatures have highlighted the disparity in antioxidant activity attributed to molecular properties, pH, and chelating ability of polysaccharides. For instance, low molecular weight polysaccharides enhanced radical scavenging as it contains relatively more terminal hydroxyl and carbonyl groups to neutralize radicals, as described by Zhang et al. (2016). Polysaccharides featuring acidic sugars may also favor hydrogen atom transfer (HAT) mechanism. In terms of pH dependence, DPPH activity was reported to diminish in acidic conditions (Kumamoto et al., 2001; Pękal & Pyrzynska, 2015). However, in acidic conditions, the polysaccharide carboxyl groups (-COOH) can act as reducing agents by donating electrons to reduce Fe^{3+} to Fe^{2+}, which favors FRAP performance (Bai et al., 2022). Besides, negatively charged or acidic polysaccharides could chelate iron ions to stabilize redox reactions, hence giving a positive effect on FRAP activity (Wang et al., 2016). Comparison of DPPH and FRAP activities of *C. nutans* with other leaf species has confirmed this trend. For instance, kenaf (*Hibiscus cannabinus* L.)

polysaccharides gave 20.55–79.77% DPPH scavenging activity and 1.26–5.08 mmol Fe^{2+}/g DW FRAP activity (Birhanie et al., 2021); mulberry leaf polysaccharides gave 17.17–44.2% DPPH scavenging activity and 0.13–0.26 mmol Fe^{2+}/g DW FRAP activity at 1 mg/mL (Ma et al., 2018).

Table 1. Bioactivities of 1 mg/mL *C. nutans* polysaccharide solution.

Bioactivities	**Results (± SD)**	**Positive Control**	**Positive Control Value (± SD)**
DPPH (1 mg/mL)	9.3 ± 0.0%	Gallic acid (1mg/mL)	81.67 ± 0.0%
FRAP	0.11 ± 0.1 mM FeSO4	Gallic acid (1mg/mL)	16.6 ± 0.4 mM FeSO4
ACE inhibition	60.8 ± 6.9%	Captopril (10 µM)	98.4 ± 0.5%
α-Glucosidase inhibition	55.3 ± 8.1%	Acarbose (1 mg/mL)	96.18 ± 1.1%
α-Amylase inhibition	No inhibition	Acarbose (1 mg/mL)	80.45 ± 1.2%
DPP4 inhibition	No inhibition	Diprotin A (0.1 mg/mL)	96.18 ± 0.1%

Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power, ACE: angiotensin I-converting enzyme, DPP4: dipeptidyl peptidase-4

3.2.2 Anti-hypertensive activity

C. nutans polysaccharide exhibited 60.8 ± 6.9% ACE inhibition at 1 mg/mL (Table 1). In comparison, the pharmaceutical reference inhibitor captopril (10 µM) achieved 98.4% inhibition under identical assay conditions. Although lower than pure captopril, achieving over 60% ACE inhibition demonstrates strong biological potential for an unpurified natural leaf carbohydrate extract without relying on synthetic pharmaceuticals. The anti-hypertensive activity of *C. nutans* polysaccharide and its underlying mechanism is further validated using in vivo model in a recent study (Chia et al., 2025). It was found that *Sprague-Dawley* rats fed with *C. nutans* polysaccharide over a fourteen-day course led to reduced systolic blood pressure and pulse wave velocity compared to their control counterparts. Further investigation revealed that the anti-hypertensive activity of *C. nutans* is ascribed to downregulation of ROS/TNF-α/MMP-9 and upregulation of eNOS/NO pathways. Previous studies found that 1 mg/mL *Gastrodia elata* Blume and *Ephedra alata* stem polysaccharides exhibited higher ACE inhibitory activities of 74.40% and 82.49%, respectively (Zhu et al., 2019; Soua et al., 2020). The difference in ACE inhibition activity could be due to the extraction conditions, whereby high temperature and long duration may degrade polysaccharide structure. Moreover, it is hypothesized that polysaccharides may inhibit ACE through different mechanisms, either by zinc chelation at the enzyme active site by negatively charged polysaccharides (Fang et al., 2019; Kaprasob et al., 2022), or pH interference by acidic polysaccharides since the optimum pH of ACE is alkaline (Soua et al., 2020). The presence of carboxylic acid or acrylic acid groups of polysaccharides may also possibly act as hydrogen bond acceptors to positively influence the inhibition of ACE (Chakraborty & Roy, 2021). Based on the current result, *C. nutans* polysaccharide has the potential as an anti-hypertensive agent. Its exact ACE inhibitory mechanism should be further investigated for therapeutic development.

3.2.2 Anti-diabetic activities

In this current study, an inhibition assay of 1 mg/mL of *C. nutans* polysaccharide solution showed that the extract inhibited up to 55.30 ± 8.1% α-glucosidase activity (Table 1). However, the extracted

polysaccharide did not inhibit α-amylase and DPP4. Comparatively, the commercial antidiabetic drug acarbose (1 mg/mL) strongly inhibited both α-glucosidase (96.18%) and α-amylase (80.45%), whereas the reference peptide diprotin A (0.1 mg/mL) inhibited DPP4 by 96.18%. These findings align with Azemi *et al.* (2020) whereby the *C. nutans* crude extract inhibited α-glucosidase activity, but contrast with two other studies whereby the methanolic extract inhibited DPP4 and α-amylase activities, respectively (Abdullah & Kasim, 2017; Murugesu et al., 2019). This could be due to variations in the extraction method used and types of polysaccharide fractions extracted. The inhibition of AG activity by *C. nutans polysaccharide* could be influenced by several factors. For instance, Wen et al. (2022) showed that low molecular weight *Huidouba* polysaccharides could enhance α-glucosidase inhibitory activity. Based on the kinetic inhibition study, the authors concluded that the number of α-(1,4) glycosidic linkages, as well as the percentage of D-glucose and D-galactose in polysaccharides were also critical to their bioactivities. Next, Dirir et al. (2022) highlighted that the number and position of hydroxyl groups may possibly be essential to α-glucosidase inhibition. Acidic pH, where the extracted polysaccharide is negatively charged, was also reported to favor α-glucosidase inhibition (Tadera et al., 2003). Nevertheless, the observed bioactivities are closely associated with the polysaccharide's physicochemical and structural properties, which will be discussed in the following section.

3.3 Physicochemical and structural properties

3.3.1 pH

The *C. nutans* polysaccharide solution at 1% (w/v) was tested to be mild acidic with a pH of 4.30 ± 0.036. This could be due to the polysaccharide containing high carboxyl groups, such as pectic polysaccharides, because of its backbone, α-(1–4)-linked D-galacturonic acid. It should also be noted that acidic polysaccharides tend to be negatively charged. Therefore, this pH condition and negative charge played a crucial role in the bioactivities aforementioned.

3.3.2 Presence of synergistic compounds

Polysaccharides are known to bind to proteins, lipids, lignin, and inorganic minerals in cells. Protein components have an effect on the antioxidant activity and give different physicochemical properties and biological activities compared to purified polysaccharides, hence potentially leading to a false conclusion (Li et al., 2019). In this study, the extracted *C. nutans* polysaccharide did not contain any traceable protein. Thus, we suggest that there was no synergistic effect from the protein content.

On the other hand, phenolics are the prominent secondary metabolites of plants with antioxidant, α-amylase, and α-glucosidase inhibitory activities (Sales et al., 2012; Lin et al., 2016). The TPC in the extracted *C. nutans* polysaccharide in this study was 1.9 ± 0.5%. Although the percentage is not high, this trace amount could act as an active component to antioxidant activity. For example, fresh white cabbage, carrot, onion, and sweet pepper contained 0.3–0.7% TPC but inhibited DPPH radicals by 22–39% (Smirnov et al., 2017). In our case, the 1.9 ± 0.5% of TPC may contribute to the significant aforementioned biological activities (Section 3.2).

Carbohydrate content provides an indication of the purity of extracted polysaccharides. The total carbohydrate content of *C. nutans* polysaccharide is 52.0 ± 14.0%, which is higher than the 43.21 ± 1.80% of hot alkali extracted sugarcane leaf polysaccharide but lower than the 83.42 ± 0.95% of mulberry leaf

polysaccharide (Zhang et al., 2016; Jiang et al., 2024). Notably, high purity does not guarantee high bioactivity. This implies a potential synergistic behavior between polysaccharide and other co-extracted biomolecules (Zhang et al., 2016).

3.3.3 Structural characteristics

Table 2 shows the sugar monomers and their compositions in *C. nutans* polysaccharide. Rhamnose was the dominant monosaccharide 77.21%, followed by galactose and glucose (9.54 and 6.41%, respectively). Other minor monosaccharides include mannose (1.33%), glucuronic acid (0.40%), and galacturonic acid (0.07%), implying the extracted polysaccharide was pectic. The presence of glucose may suggest co-extraction of non-pectic polysaccharides in an acidic environment (Methacanon et al., 2014).

Table 2: Sugar monomers and their compositions in extracted *C. nutans* polysaccharide.

Monosaccharides	Concentration (mg/mL) (± SD)	Percentage (%)
Glucuronic acid	0.06 ± 0.01	0.40
Mannose	0.23 ± 0.05	1.33
Galacturonic acid	0.01 ± 0.021	0.07
Ribose	0.00 ± 0.00	0.00
Rhamnose	12.40 ± 5.44	77.21
Glucose	1.03 ± 0.69	6.41
Galactose	1.53 ± 0.36	9.54

The presence of a high amount of rhamnose could indicate a high degree of branching. The linearity value calculated based on the equation (GalA+Rham) / (Fuc+Rham+Ara+Gal) shows the results of 0.8420, with rhamnose as the backbone of the polysaccharides. The high abundance of rhamnose compared to galacturonic acid content could also indicate that *C. nutans* polysaccharide extract mainly comprised of rhamnogalacturonan I (RGI) domains (Cornuault et al., 2018; Kaczmarska et al., 2022). The ratio of neutral sugars to rhamnose roughly indicates the length of the side chains (Sengkhamparn et al., 2009), in which the common side chain attached to the rhamnose residue of RGI can be either branched or unbranched oligosaccharides including galactose, arabinose (Jiang et al., 2021), and/or arabinogalactans (Yapo et al., 2007). The RGI side chain may also contain other glycans such as mannose, fucose, and galactose (Naran et al., 2008; Atmodjo et al., 2013). Although direct molecular weight determination via size-exclusion or gel permeation chromatography was not accessible for this preliminary screening, structural parameters can be inferred from the sugar distribution. The ratio of (arabinose, galactose and mannose) to rhamnose was 0.2051 would suggest that the *C. nutans* polysaccharide contains shorter side chains compared with Okra pod polysaccharide, in which the value is around 1.0 (Sengkhamparn et al., 2009). However, absolute chain lengths and definitive molecular weight profiles must be validated using direct chromatographic techniques in future structural studies.

Fig. 1 shows the IR spectrum of *C. nutans* polysaccharide. The weak and broad absorption band at 3600–3200 cm^{-1} indicated the stretching vibration of the hydroxyl group (O-H), which could be related to intra- and intermolecular hydrogen bonds of the galacturonic acid polymer (Singthong et al., 2004). At the 2000–1500 cm^{-1} region, a C=O ester stretching was observed as shoulders at around 1718 cm^{-1}, which

could be linked to the presence of uronic acid in the polysaccharide (Wang et al., 2017). A stronger absorption at 1616 cm^{-1} could be the methyl esterified carboxyl group (COO-R) stretching vibration of ionic carboxyl groups (COO^-) (Kozioł et al., 2022). The ionic carboxyl groups (COO^-) can also act as antioxidants by forming hydrogen bonds with free radicals. The phenolic groups were observed at absorption bands between 1580–1530 cm^{-1}. Bands at 1720, 1616, 1450–1440 and 1078 cm^{-1} indicate the characteristic of pectin (Hong et al., 2021). The fingerprint region for pectin shows a weaker symmetric (COO^-) stretching followed by moderately intense absorption patterns between 1300 and 800 cm^{-1} (Singthong et al., 2004). The multiple bands at region 920–840 cm^{-1} are also associated with fingerprint region for β-type or α-(1→6) glycosidic bonds of carbohydrates (Wu et al., 2020; Hong et al., 2021). Band between 1200 and 1100 cm^{-1} was from ether R–O–R and cyclic C–C bonds in the ring structure of pectin could indicate the existence of pyranose forms (Liu et al., 2010). The spectral range at 1100–930 cm^{-1} is associated with glycosidic bonds and ring vibrations of pectin (Kozioł et al., 2022). The band at 1078 cm^{-1} might suggest the presence of galactose. The IR bands of β-(1→ 6)- or β-(1→3)-linked galactan occur at 1078–1072 cm^{-1} (Kacuráková et al., 2000). Based on the peak areas in 1718 and 1616 cm^{-1}, esterification degrees (DE) of the extracted polysaccharide were calculated. The DE value was 14%, which is considered low (DE<50) (Güzel & Akpınar, 2019), aligning with pectins from the leaves of *Caesalpinia pulcherrima* (30.1%), *Premna Oblongifolia* Merr. (48.01%) and *Hedera nepalensis* (6.1–24.4%) (Elsyana & Alvita, 2021; Melo et al., 2022; Ho et al., 2023). Nonetheless, Li et al. (2022) showed that different DE of okra pectic-polysaccharides (4.77–42.13%) was not related to antioxidant activity.

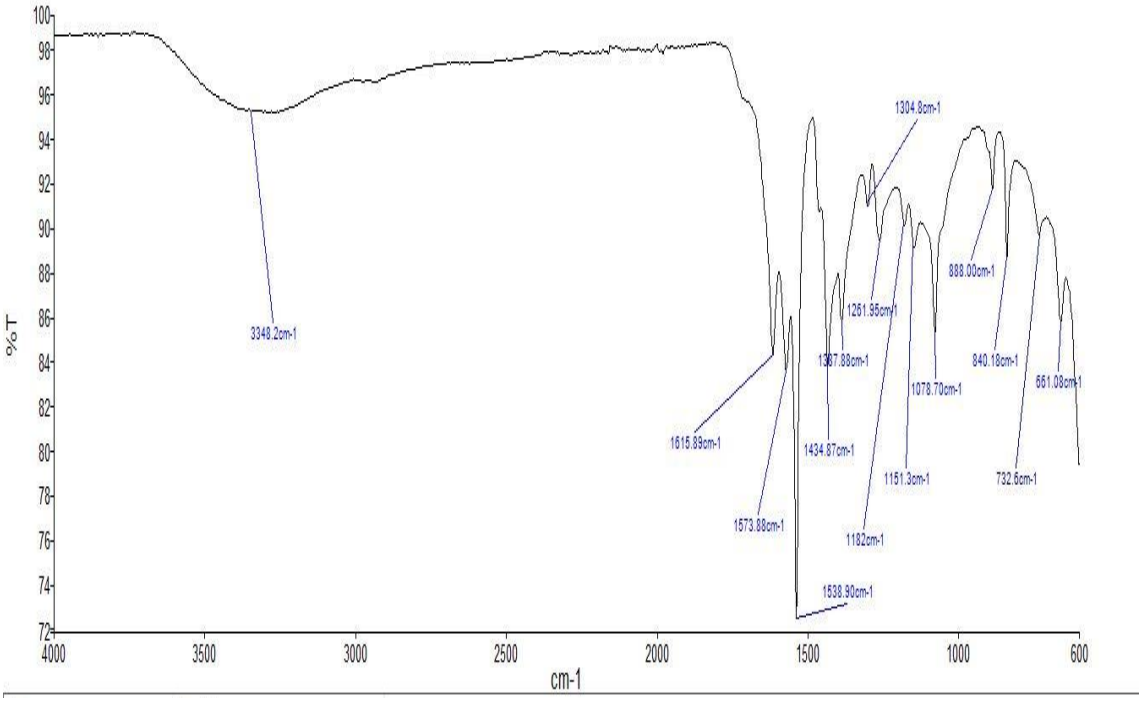

Fig. 1. IR spectrum of *C. nutans* polysaccharide

4. CONCLUSION

Overall, *C. nutans* polysaccharide is a pectic-polysaccharide with small branch size and low DE. These structural characteristics could facilitate interactions with Fe^{3+}, ACE and α-glucosidase. Its mild acidic property and phenolic components enhanced bioactivity. This study, therefore, unveiled the physicochemical profiles and potential for future pharmaceutical or nutraceutical development after additional mechanistic and in vivo validation of the bioactive pectic-polysaccharide from *C. nutans* leaves. Despite the promising outcomes, it is important to acknowledge the limitations of this study. Since this study represents an initial characterization, the bioactivities were evaluated at a single concentration without IC_{50} determination. Future studies should include concentration-dependent assays to establish IC_{50} values. Further investigation is also needed to determine the actual mechanism between polysaccharide and its bioactivities. More in-depth experiments such as binding studies, cell culture and *in vivo* experiments are required to validate the mechanisms underlying the polysaccharide's bioactivity. Structural analysis techniques such as mass spectrometry, X-ray crystallography and nuclear magnetic resonance (NMR) are required for advanced structural analysis. In conclusion, this research bridges the gap between phytochemicals studies by expanding the research scope of *C. nutans*, as a strategy in maximizing the usage of this plant.

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CONFLICT OF INTEREST STATEMENT

The authors declared that they have no conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

Ainolsyakira Mohd Rodhi: Investigation, Data curation, Formal analysis, Writing- Original draft. **Yong Chia Tan**: Investigation, Data curation, Formal analysis, Writing- Original draft. **Muhammad Hakimin Shafie**: Validation, Supervision, Writing - Review & Editing. **Olalere Olusegun Abayomi**: Methodology, Writing - Review & Editing. **Pei-Gee Yap**: Methodology, Writing - Review & Editing. **Yvonne Yve Wen Thoe:** Formal analysis, Writing - Review & Editing. **Chee-Yuen Gan**: Conceptualization, Funding acquisition, Supervision, Writing - Review & Editing.

DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT (OpenAI) solely for language refinement and improvement of manuscript readability. The authors reviewed and edited all generated content and take full responsibility for the content of this publication.

ETHICS STATEMENT

Not applicable.

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