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# Phytochemical profiling, antioxidant activity, and structural elucidation of bioactive compounds from anaerobically digested cow dung slurry

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## Abstract

**Introduction** Anaerobic digestion (AD) is heralded as a cornerstone of sustainable waste management, converting organic residues into valuable biogas. While biogas production from AD is well-documented, the valorisation of anaerobic digestate, particularly for its pharmaceutical applications, remains underexplored. We analysed aqueous ethanolic (70%) and methanolic (50%) extracts from cow dung slurry (CSW) to characterise their bioactive compounds, antioxidant capacity, and structural properties.

**Results** Qualitative and quantitative phytochemical analyses revealed a diverse array of secondary metabolites, including phenolics, flavonoids, tannins, alkaloids, terpenoids, glycosides, and saponins, whereas phlobatannins, volatile oils, and resins were undetectable. Notably, the ethanol extract exhibited significantly higher concentrations of total phenolics (~ 250 µg catechin equivalents [CE]/mL) and flavonoids (~ 220 µg CE/mL) than the methanol extract ($p < 0.0001$), while other classes, such as saponins, tannins, flavanols, and proanthocyanidins, were present at lower and comparable levels across solvents ($p > 0.05$). Fourier-transform infrared spectroscopy (FTIR) analysis indicated a higher abundance of phenolic (O-H stretching at 3450.53 $\text{cm}^{-1}$) and carboxyl (C = O at 1778.40 $\text{cm}^{-1}$) groups in the methanol extract, suggesting strong antioxidant potential. In contrast, the ethanol extract contained alkyl (2982.51 $\text{cm}^{-1}$), nitro (1588.02 $\text{cm}^{-1}$), and halogenated (652.35 $\text{cm}^{-1}$) functional groups. Nuclear magnetic resonance (NMR) spectroscopy further identified key secondary metabolites, including harmine, salicylic acid, anacardic acid, quercetin, and gallic acid. In vitro, free radical scavenging assays demonstrated concentration-dependent antioxidant activity, with the methanol extract consistently outperforming the ethanol extract ($p < 0.05$). At 800 µg/mL, the methanol extract exhibited near-comparable activity to gallic acid ($p = 0.0927$).

**Conclusion** These findings suggest that CSW-derived methanol extract holds significant promise as a natural source of bioactive compounds with potential pharmaceutical and nutraceutical applications.

**Keywords** Valorisation of anaerobic organic waste, Metabolite profiling, Free radical scavenging, Natural product discovery, FT-IR and NMRcharacterisation

## 1 Introduction

Anaerobic digestion (AD) is a cornerstone technology in waste management and renewable energy production, leveraging complex microbial consortia to convert organic waste into valuable end-products, such as biogas, bio-fertilisers and other value-added products [1].

Beyond its established role in energy recovery, the AD microbiome represents an underexplored reservoir of microbial diversity with potential for the discovery of novel bioactive compounds [2, 3]. Despite advances in understanding AD's microbial ecology and process optimisation [4, 5], the therapeutic potential of its liquid byproduct, bio-slurry, remains largely untapped – a critical gap this study addresses.

The AD process progresses through four metabolic stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis), each mediated by specialised microbial populations [6, 7]. While the primary outputs (biogas, digestate, and bio-slurry) are well-characterised for applications in energy and agriculture [8–10], the bio-slurry's chemical complexity, enriched with microbial secondary metabolites, biopolymers, and recalcitrant organic fractions, suggests untapped pharmacological value. Recent bioprospecting efforts have prioritised plant and marine ecosystems [11–14]. Yet, the extreme anaerobic environment of AD systems, with its unique microbial stress responses and metabolic adaptations, could yield structurally distinct bioactive compounds.

Emerging evidence supports this premise: Olaniyan and Ajayi [15] identified AD's stationary-phase microbes as promising sources of antimicrobial metabolites, while Lu et al. [17]. linked anaerobic degradation products (e.g., steroids, lignin derivatives) to disease-resistant properties in digestate. Similarly, cow dung, a common AD feedstock, has shown antimicrobial activity attributable to its microbial constituents [16]. However, systematic exploration of bio-slurry's therapeutic potential is lacking, particularly regarding its small-molecule metabolites and their mechanistic actions [19].

This study pioneers the valorisation of AD-derived bio-slurry as a possible source of antimicrobial and anti-cancer agents, bridging the gap between waste valorisation and drug discovery. By combining phytochemical characterisation, in vitro bioassays, and advanced spectroscopic techniques (FT-IR, NMR), we identify bioactive constituents in hydro-methanol and hydro-ethanol extracts of bio-slurry, leveraging solvent-specific fractionation to target diverse metabolites, evaluate therapeutic potential through antioxidant, correlating activity with phytochemical profiles and propose a sustainable pipeline for repurposing AD waste into high-value therapeutics, aligning with circular bioeconomy goals.

However, integrating cultivation-independent metabolite profiling with functional assays presents a novel approach to uncovering the hidden pharmacological potential in anaerobic waste streams. This study is a timely contribution, given the urgent need for new pharmaceuticals [18, 19].

## 2 Materials and methods

### 2.1 Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade. Methanol (≥ 99.8%), ethanol (≥ 99.5%), aluminium chloride hexahydrate ($AlCl_3 \cdot 6\ H_2O$, ≥ 98%), sodium nitrite ($NaNO_2$, ≥ 99%), Folin-Ciocalteu reagent, gallic acid (≥ 98%), catechin (≥ 98%), quercetin (≥ 95%), sodium acetate (≥ 99%), ammonium sulfate (≥ 99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH, ≥ 95%), ascorbic acid (≥ 99%), Griess reagent, thiobarbituric acid (TBA, ≥ 98%), ferrous sulfate ($FeSO_4$, ≥ 99%), ethylenediaminetetraacetic acid (EDTA, ≥ 99%), potassium bromide (KBr, ≥ 99%), deuterated water ($D_2O$, 99.9% D), 3-(trimethylsilyl)propionic acid sodium salt (TSP, ≥ 98%), hydrogen peroxide ($H_2O_2$, 30% w/v), and Fehling's solution were purchased from Sigma-Aldrich (USA), Merck (Germany), Fisher Scientific (UK), Cambridge Isotope Laboratories (USA), or GE Healthcare Life Sciences (USA). Whatman No. 1 filter paper was used for filtration. All chemicals were stored under recommended conditions and used following standard analytical protocols.

### 2.2 Sample collection

About 10 kg of fresh cow dung was sourced from a Fulani cow settlement in Osogbo, Osun State, within the geographical coordinates of Latitude: $7°45'0''N$ – 7°55′0″N and Longitude: 4°30′0″E – 4°40′0″E, covering approximately 10 km of urban area. New polyethene nylon and gloves were collected to ensure minimal contamination. The Fulani lairage housed approximately 100 local cows of various breeds commonly found in northern Nigeria, such as Red Bororo/Mbororo, Bunaji, Muturu, Sokoto Gudali, and N'Dama.

### 2.3 AD process of bio-slurry

The digestion process followed the methods outlined by Olaniyan et al. [20]. and Bamigboye et al. [21]. with slight modifications and lasted for three months. The collected dung was mixed with water in a 1:2 w/v ratio within a 200-litre bucket, and all shaft or plant remnants were removed. This mixture was subsequently transferred to a locally fabricated biodigester for AD within a mesophilic temperature range.

### 2.4 Extraction of bio-slurry

After AD, the bio-slurry was collected from the bioreactor outlet and filtered using Whatman No. 1 filter paper. The filtrate underwent cold maceration in separate solutions of 50% hydro-methanol and 70% hydro-ethanol for 72 h each, and was then filtered. All samples were meticulously labelled for proper identification. Finally, the crude hydro-extracts were concentrated using a Büchi® Model R-200 rotary evaporator under reduced pressure at 40 °C. The concentrated extracts were then stored at -20 °C until further analysis.

### 2.5 Phytochemical properties of bio-slurry extract

#### *2.5.1 Qualitative photochemical*

The qualitative phyto-compounds presented in the hydro-methanol and hydro-ethanol bio-slurry extracts were performed using standard procedures as described by Harborne [70], Trease and Evans [71], and Archana et al. [72]. , with slight modifications.

Anthraquinones were detected by adding 2% diluted hydrochloric acid to 1 mg of extract, where the appearance of a red colour was considered indicative. The presence of phenolic compounds was assessed by dissolving 1 mg of extract in 2 ml of distilled water, followed by the addition of 10% ferric chloride, which produced a characteristic green or blue colour. Similarly, coumarins were identified when 1 mg of extract reacted with 1 ml of 10% sodium hydroxide, producing a yellow colour, while flavonoids were detected by the addition of ferric chloride to 1 ml of extract, forming a blackish-red precipitate.

Saponins were evaluated by vigorously shaking 2 mg of extract with 2 ml of distilled water for approximately 15 min, after which the formation of a stable frothy layer was interpreted as positive. Glycosides were tested by mixing 1 ml of extract with 1 ml of glacial acetic acid and cooling, followed by addition of two drops of ferric chloride and careful layering of concentrated sulphuric acid; a reddish-brown ring at the interface was taken as evidence of glycosides. The appearance of pink precipitates upon addition of 10% ammonia to 1 ml of extract indicated the presence of phlobatannins.

To determine steroids and phytosteroids, 1 ml each of chloroform and concentrated sulphuric acid were added to 1 ml of extract, resulting in a brown ring and a bluish-brown ring, respectively. Tannins were identified by adding 2 ml of 5% ferric chloride to 1 mg of extract, which produced a greenish-black or dark blue colour. Vitamin C was detected by reacting 1 ml of extract with dinitrophenylhydrazine dissolved in concentrated sulphuric acid, forming yellow precipitates. Quinones were confirmed when 1 ml of extract reacted with concentrated sulphuric acid to produce a red colour.

Alkaloids were assessed by treating 2 ml of extract with concentrated hydrochloric acid and Mayer's reagent, where the development of a green colour or white precipitate indicated their presence. Terpenoids were tested by adding 2 ml each of chloroform and concentrated sulphuric acid to 0.5 mg of extract, producing a brown interface layer. Oils and resins were qualitatively confirmed by spotting the extract on filter paper and observing a translucent appearance afterdrying.

#### *2.5.2 Quantitative phytochemical*

The total flavonoid content of the CSW extract was determined using the aluminium chloride/sodium nitrite colourimetric method of Park et al. [22]. with slight modifications. Briefly, a reaction mixture was prepared in a test tube by combining 0.3 mL of the extract, 0.15 mL of 0.5 M $NaNO_2$ solution, 0.3 mL of 0.3 M $AlCl_3{\cdot}6H_2O$ solution, and 3.4 mL of 30% methanol. After incubation for 5 min at room temperature, 1 mL of 1 M NaOH was added. The absorbance was then measured at 506 nm using a Multicell Peltier Module G9889A for an Agilent Cary 3500 UV-Vis Spectrophotometer. A standard curve was developed using known amounts of a reference flavonoid (0-100 mg/mL). The total phenolic content in the CSW extracts was determined using the Folin-Ciocalteu method [23], with the results expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight extract or fraction. The content of total proanthocyanidins in the extracts was determined using the saturated ammonium sulfate solution $(NH_4)_2SO_4$ method described by Vigneault et al. [24]. , and results were expressed as catechin equivalents (CE) per unit volume of the extract.

Van Buren and Robinson's [25] method was modified to determine the tannin content of the CSW extracts. The results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight extract. The total flavonols in the extracts were evaluated using a sodium acetate solution and an $AlCl_3$ solution, following the method of Kumaran and Karunakaran [26]. The total flavonoid content of the extracts was reported as milligrams of quercetin equivalents (mg QUE) per gram of dry-weight extract based on this calibration curve. The presence of reducing sugars in the extracts was qualitatively assessed using Fehling's solution described by Djaafar and Ridha [27].

### 2.5.3 Characterisation of crude extract using FT-IR and NMR

The methods used to characterise the functional groups and potential bioactive compounds present in the raw and carbonised bio-slurry extracts are;

### 2.5.4 Fourier-transform infrared (FT-IR) spectroscopy

The extracts were mixed with potassium bromide (KBr) at a 1:100 ratio (sample: KBr) to prepare tablets for analysis. FT-IR spectra were recorded using a Varian 660 MidIR Dual MCT/DTGS Bundle spectrometer with an ATR accessory. The scan range was set from 4000 $cm^{-1}$ to 500 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and 200 scans per sample. Functional group identification was achieved by analysing the obtained spectra.

### 2.5.5 Nuclear magnetic resonance (NMR) spectroscopy

$^1H$ and $^{13}C$ NMR spectra were acquired using a Bruker Advance 600 III spectrometer (operating frequencies: 600.18 MHz for $^1H$ and 150.93 MHz for $^{13}C$). For $^1H$ NMR analysis, 10 mg of lyophilised hydro-alcoholic extract was dissolved in 600 μL of deuterated water ($D_2O$) containing 0.1 mM 3-(Trimethylsilyl)propionic acid sodium salt (TSP) as a reference standard. Samples were vortexed, centrifuged, and transferred to NMR tubes. Spectra were recorded for all samples, and standard compounds (quercetin, ferulic acid, and gallic acid) were prepared in a similar manner. Standard acquisition parameters were: room temperature (300 K), 256 scans, noesygppr1d pulse sequence, acquisition time 0.82 s, relaxation delay 3 s, spectral width 10,000 Hz. Free induction decay (FID) data were zero-filled to 32 K data points before processing.

Raw $^1H$ NMR spectra were baseline corrected, phased, and calibrated using TOPSPIN software (Version 3.6.0). Post-processed NMR data were imported into MATLAB (Version R2014a) for spectral alignment using a recursive segment-wise peak alignment method. Regions containing water signals were removed. Processed NMR data were exported from MATLAB and combined with bioassay results for further analysis using SIMCA software (Version 14.1).

### 2.5.6 In vitro free radical scavenging evaluation of the extracted bio slurry

The antioxidant activities of the CSW extracts were assessed using various established methods, with results expressed as micrograms per millilitre (μg/mL) of ascorbic acid and gallic acid equivalents. The nitric oxide (NO) radical scavenging activity was evaluated using the Griess reagent method described by Bhaskar and Balakrishnan [28]. Hydroxyl radical scavenging activity was determined following the protocol of Guchu et al. [29]. The iron/EDTA/$H_2O_2$ complex method, as outlined by Klein et al. [30], was employed to evaluate hydroxyl radical scavenging. In contrast, hydrogen peroxide scavenging activity was assessed using the method described by Gülçin et al. [31]. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured using the approach detailed by Awojide et al. [32]. Additionally, the inhibition of ferrous sulfate-induced lipid peroxidation was evaluated using the thiobarbituric acid reactive substances (TBARS) assay, as described by Fatoki et al. [33]. All antioxidant activities were consistently expressed as μg/mL of ascorbic acid and gallic acid equivalents.

### 2.6 Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD) ($n = 3$). Statistical analysis was performed using one-way analysis of variance (ANOVA) in GraphPad Prism version 10.3.1. Differences between groups were assessed at a significance level of $p < 0.05$, and post hoc comparisons were carried out using the least significant difference (LSD) test to identify homogeneous groups. P-values are provided within the figures or in the corresponding figure legends to denote statistical significance compared to the control group.

## 3 Results

### 3.1 Qualitative phytochemicals

As shown in Table 1, the phytochemical analysis of CSW extracts revealed the presence of diverse bioactive compounds in both ethanol and methanol extracts, including anthraquinones, phenols, coumarins, flavonoids, saponins, glycosides, xanthoproteins, sterols, phytosterols, tannins, vitamin C, quinones, alkaloids, and terpenoids. Notably, both extracts lacked phlobatannins, volatile oils, and resins, indicating a distinct metabolite profile.

Figure 1 summarises the quantitative phytochemical analysis of the ethanol and methanol extracts of CSW from AD. Phytochemical analysis of CSW extracts revealed solvent-dependent variations in bioactive compounds. All differences were statistically significant ($p < 0.05$).

The quantitative analysis of selected phytochemicals in ethanol and methanol extracts of CSW is presented in Fig. 1. Among the phytochemicals assessed, TPC was the most abundant, with the ethanol extract yielding significantly higher levels (approximately 250 μg catechin equivalents [CE]/mL) compared to the methanol extract (approximately

**Table 1** Qualitative phytochemical analysis of the extract of AD of CSW

| Phytochemicals | CSW ethanol | CSW methanol |
|---|---|---|
| Anthraquinone | + | + |
| Phenols | + | + |
| Coumarins | + | + |
| Flavonoids | + | + |
| Saponins | + | + |
| Glycosides | + | + |
| Phlobotannins | – | – |
| Sterols | + | + |
| Phytosterols | + | + |
| Tannins | + | + |
| Vitamin C | + | + |
| Quinones | + | + |
| Alkaloids | + | + |
| Terpenoids | + | + |
| volatile oil | – | – |
| Resins | – | – |

Legend: + = present, – = absent

Quantitative Phytochemical Analysis

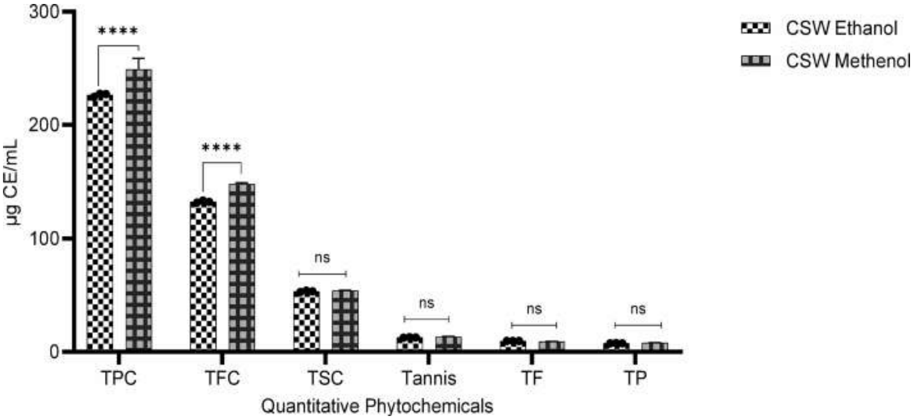

**Fig. 1** The Quantitative Phytochemical analysis of the extract of AD of CSW (µg CE/mL). *PC* Total phenolic contents, *TFC* Total flavonoid contents, *TSC* Total sugar content, *TF* Total flavonols, and *TP* Total proanthocyanin.

**Table 2** FT-IR absorption frequencies of functional groups in the ethanolic extract of anaerobically digested CSW

| Peak wavelength ($cm^{-1}$) | Transmittance (%) | Assignment | Functional group |
|---|---|---|---|
| 3450.00 | 24.60 | –OH, stretching absorption that is due to inter- and intramolecular hydrogen bonds | Hydroxyl |
| 2982.51 | 31.72 | C-H stretching and bending vibrations of CH, $CH_2$, and $CH_3$ | Alkane |
| 2450.41 | 72.54 | -C-O stretching and bending vibrations | Carbonyl |
| 1877.32 | 52.08 | C = C stretching Vibrations in aromatic rings | Alkenes |
| 1792.49 | 67.15 | C = O stretching vibration | Carboxyl |
| 1588.02 | 76.98 | $–NO_2$ aliphatic nitro groups, C–N stretching of aliphatic amines, and C–O stretching of carboxylic groups. | Carboxyl/ amines |
| 1250.61 | 52.16 | C = O stretching in vibration | Polysaccharide |
| 850.97 | 80.20 | C = C stretching vibration | Aromatics |
| 652.35 | 71.53 | C-Cl, C-Br, and C-I stretching vibration | Alkyl halide |

220 µg CE/mL) ($p < 0.0001$). Similarly, the TFC was significantly greater in the ethanol extract than in the methanol extract ($p < 0.0001$).

Conversely, no significant differences were observed between ethanol and methanol extracts for TSC, tannins, TF, or TP (ns, $p > 0.05$ in all cases). These latter phytochemicals were present at much lower concentrations relative to TPC and TFC, irrespective of the extraction solvent.

### 3.2 Functional group analysis by FT-IR spectroscopy

The FTIR spectroscopic analysis of the ethanolic extract of cassava biogas slurry, as shown in Table 2; Fig. 2A, revealed the presence of diverse functional groups. A broad and intense absorption band at 3450.53 $cm^{-1}$ indicated O–H stretching vibrations characteristic of hydroxyl groups, suggesting the presence of alcohols and phenolic compounds. Peaks observed at 2961.33 $cm^{-1}$ and 2900.07 $cm^{-1}$ corresponded to C–H stretching vibrations of aliphatic hydrocarbons. Sharp absorption bands at 1777.31 $cm^{-1}$ and 1670.63 $cm^{-1}$ were assigned to C = O stretching vibrations, indicative of carbonyl functional groups, including esters and conjugated ketones. The band at 1620.58 $cm^{-1}$ suggested C = C stretching of aromatic rings, while the peaks at 1376.48 $cm^{-1}$, 1261.35 $cm^{-1}$, 1154.71 $cm^{-1}$, and 1051.39 $cm^{-1}$ confirmed the presence of C–H bending

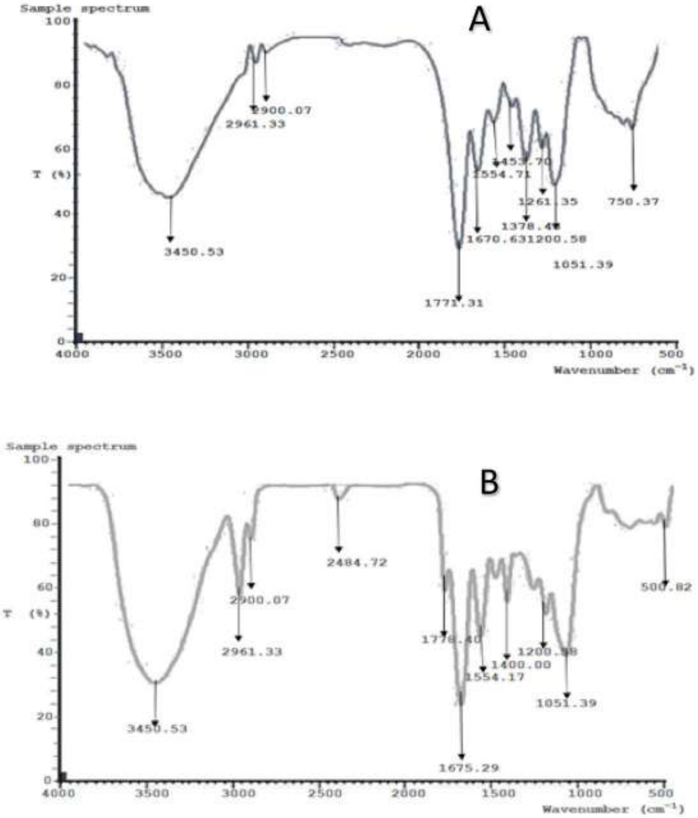

**Fig. 2** Showing the FT-IR Chromatograph of **A** the functional groups in the crude hydro-ethanol of CSW extract, with the high peak of glutamic acid and **B** the crude hydro-methanol of CSW extract, with the high peak of glutamic acid

**Table 3** FT-IR absorption frequencies of functional groups in the methanolic extract of anaerobically digested CSW

| Peak wavelength (cm$^{-1}$) | Transmittance (%) | Assignment | Functional group |
|---|---|---|---|
| 3450.53 | 32.50 | O-H, N-H stretching vibration absorption of phenolic groups. | Phenolic and hydroxyl |
| 2961.33 | 58.62 | C–H symmetric stretching vibration of aromatic overtone due to aromatic amino acids | Alkenes |
| 2900.07 | 72.64 | C = O stretching vibration | Carboxyl |
| 2484.72 | 38.91 | N–H weak stretching vibration of the amide I group | Amide |
| 1778.40 | 60.00 | C = O stretching vibration | Carboxyl |
| 1675.29 | 24.31 | N–H stretching in primary amines and amides | Amide |
| 1554.17 | 45.62 | -OH, bending vibrations of the hydroxyl group. | Hydroxyl |
| 1400.00 | 58.15 | Glucose ring stretching | Glucose |
| 1200.58 | 52.66 | C-H bending vibration | Ketone |
| 1051.39 | 40.00 | C = O stretching in vibration | Polysaccharide |
| 500.82 | 80.03 | C-H bending (2 adjacent free H's) | Alkanes |

and C–O stretching typical of esters, ethers, and alcohols. The presence of aromatic C–H out-of-plane bending was evident by a strong band at 750.37 cm$^{-1}$.

While the methanolic extracts also contain (as shown in Table 3; Fig. 2B) a broad and intense absorption band at 3450.53 cm$^{-1}$, indicating O–H stretching vibrations, which confirms the presence of hydroxyl groups typical of alcohols and phenolic compounds.

Peaks observed at 2961.33 $cm^{-1}$ and 2900.07 $cm^{-1}$ corresponded to the aliphatic structure's C–H stretching vibrations. Distinct absorption bands at 1777.31 $cm^{-1}$ and 1670.63 $cm^{-1}$ were assigned to C = O stretching of carbonyl groups, suggesting the occurrence of esters, ketones, or amides. The band at 1620.58 $cm^{-1}$ reflected the C = C stretching of aromatic systems. Furthermore, strong C–O stretching and bending vibrations were detected at 1376.48 $cm^{-1}$, 1261.35 $cm^{-1}$, 1154.71 $cm^{-1}$, and 1051.39 $cm^{-1}$, characteristic of esters, ethers, or alcohols. A sharp absorption at 750.37 $cm^{-1}$ indicated C–H out-of-plane bending vibrations in aromatic compounds.

### 3.3 Nuclear magnetic resonance (NMR) of the CSW slurry extracts

The NMR spectra of the hydro-ethanol and hydro-methanol extracts revealed diverse secondary metabolite groups with the high peak of glutamic acid, as shown in Tables 4 and 5, respectively.

### 3.4 In vitro free radical scavenging activities of bio-slurry extracts

The free radical scavenging capacity of bio-slurry extracts was evaluated in vitro using established antioxidant assays. As shown in Fig. 3, both hydro-methanol and

**Table 4** NMR spectrum interpretation of secondary metabolites present in the hydro-ethanol extract of AD of CSW

| S/*N* | Metabolites | Group | δH (Multiplicity) |
|---|---|---|---|
| 1. | Harmine | $CH_2$ | 9.60 (s) |
| | | $CH_2$ | 9.74 (s) |
| | | CH | 9.82 (s) |
| 2. | Salicylic Acid | $CH_3$ | 9.20 (s) |
| | | $CH_2$ | 9.23 (s) |
| | | H. OH | 9/46 (s) |
| | | 1 H, C(=O) OH | |
| 3. | Anacardic acid | $CH_3$ | 8.53 (d) |
| | | CH | 8.55 (d) |
| 4. | Cardol | $CH_3$ | 5.75 (d) |
| | | $CH_2$ | 5.81 (d) |
| | | 2 H. OH | 5.86 (d) |
| 5. | Asparagine | $CH_2$ | 4.40 (s) |
| | | $CH_2$ | 4.51 (s) |
| | | CH | 4.68 (s) |
| 6. | Gallic acid | $CH_2$ | 5.10 (d) |
| | | CH | 5.32(q) |
| 7. | Glutamic acid | $CH_3$ | 3.63 (d) |
| | | $CH_2$ | 3.69 (d) |
| | | $CH_2$ | 3.70 (m) |
| 8. | Quercetin | $CH_3$ | 3.20 (m) |
| | | $CH_2$ | 3.25 (m) |
| 9. | Cardanol | $CH_3$ | 2.81 (m) |
| | | $CH_2$ | 2.86 (m) |
| | | 3 $H.CH_3$ | 2.90 (m) |
| 10. | P-Cymene | $CH_3$ | 2.35 (d) |
| 11. | Tyrosine | $CH_3$ | 2.37 (d) |
| | | $CH_2$ | 2.42 (d) |
| | | CH | 2.48 (d) |
| 12. | D-germacrene | $CH_3$ | 1.38 (d) |
| | | $CH_2$ | 1.45 (d) |
| | | $OCH_2$ | 1.47 (m) |
| 13. | Alanine | CH3 | 0.65 (q) |
| | | $CH_2$ | 0.71 (q) |
| | | CH | 0.77 (q) |

**Table 5** NMR spectrum interpretation of secondary metabolites present in the hydro-methanol extract of AD of CSW

| S/*N* | Metabolites | Group | δH (Multiplicity) |
|---|---|---|---|
| 1 | Harmine | $CH_2$ | 9.22 (s) |
| | | $CH_2$ | 9.28 (s) |
| | | CH | 9.37 (s) |
| 2 | Salicylic Acid | $CH_3$ | 9.00 (s) |
| | | $CH_2$ | 9.06 (s) |
| | | H. OH | 9.13 (s) |
| | | 1 H, C(=O) OH | |
| 3 | Anacardic acid | $CH_3$ | 8.25 (d) |
| | | CH | 8.33 (d) |
| 4 | Cardol | $CH_3$ | 5.60 (d) |
| | | $CH_2$ | 5.63 (d) |
| | | 2 H. OH | 5.71 (d) |
| 5 | Asparagine | $CH_2$ | 4.40 (s) |
| | | $CH_2$ | 4.51 (s) |
| | | CH | 4.68 (s) |
| 6 | Gallic acid | $CH_2$ | 4.90 (d) |
| | | CH | 5.00 (q) |
| 7 | Glutamic acid | $CH_3$ | 3.50 (d) |
| | | $CH_2$ | 3.54 (d) |
| | | $CH_2$ | 3.63 (m) |
| 8 | Quercetin | $CH_3$ | 3.00 (m) |
| | | $CH_2$ | 3.21 (m) |
| 9 | Cardanol | $CH_3$ | 2.73 (m) |
| | | $CH_2$ | 2.81 (m) |
| | | 3 H.$CH_3$ | 2.87 (m) |
| 10 | P-Cymene | $CH_3$ | 2.30 (d) |
| 11 | Tyrosine | $CH_3$ | 1.90 (d) |
| | | $CH_2$ | 1.97 (d) |
| | | CH | 2.03 (d) |
| 12 | D-germacrene | $CH_3$ | 1.38 (d) |
| | | $CH_2$ | 1.45 (d) |
| | | $OCH_2$ | 1.47 (m) |
| 13 | Alanine | CH3 | 0.65 (q) |
| | | $CH_2$ | 0.71 (q) |
| | | CH | 0.77 (q) |

s = singlet, d = doublet, t = triplet, dd = double-doublet, dt = double-triplet m = multiplet

(See figure on next page.)

**Fig. 3** Scavenging activities of the hydro-extracts compared to control (ascorbic and gallic acid) across concentrations (50, 100, 200, 400, and 800 µg/mL), shows that **A** At 50 µg/mL, gallic acid exhibited the highest scavenging activity, significantly outperforming all other treatments. CSW methanol showed slightly better activity than CSW ethanol ($p = 0.0195$), while ascorbic acid demonstrated comparable activity to CSW methanol. These trends were consistent at higher concentrations, with gallic acid remaining superior across all levels. **B** At 100 µg/mL, gallic acid significantly surpassed CSW ethanol and ascorbic acid in scavenging activity ($p < 0.0001$). CSW methanol consistently showed higher activity than CSW ethanol ($p = 0.0202$), while ascorbic acid demonstrated moderate activity, remaining between the performance of the two extracts. **C** At 200 µg/mL, gallic acid maintained its superior antioxidant activity ($p < 0.0001$), followed by ascorbic acid, which outperformed CSW Ethanol ($p = 0.0175$). CSW methanol exhibited significantly greater activity than CSW ethanol ($p = 0.0029$), confirming its enhanced efficacy over the ethanol extract at this concentration. **D** At 400 µg/mL, gallic acid continued to exhibit the highest scavenging activity ($p < 0.0001$). CSW methanol outperformed CSW Ethanol ($p = 0.0112$), highlighting its consistent effectiveness across higher concentrations. Ascorbic acid showed intermediate activity, maintaining its position relative to the extracts and gallic acid. **E** At 800 µg/mL, gallic acid retained the highest scavenging activity ($p < 0.0001$), while CSW methanol showed slightly higher activity than CSW Ethanol ($p < 0.0001$). No significant differences were observed between CSW methanol and gallic acid ($p = 0.0927$), indicating a narrowing gap in activity levels at this concentration. Ascorbic acid performed moderately, surpassing both hydro-extracts in some cases

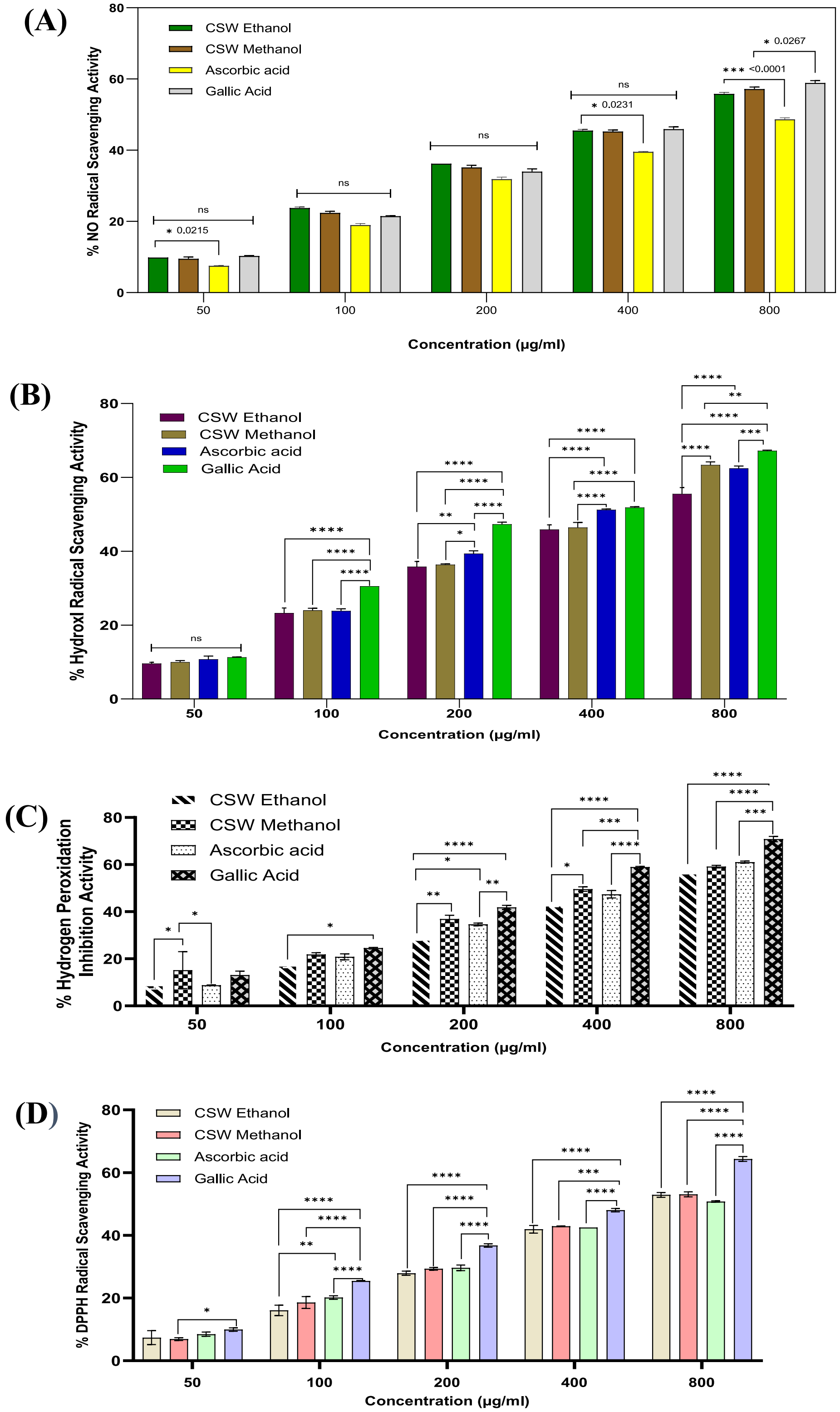

**Fig. 3** (See legend on previous page.)

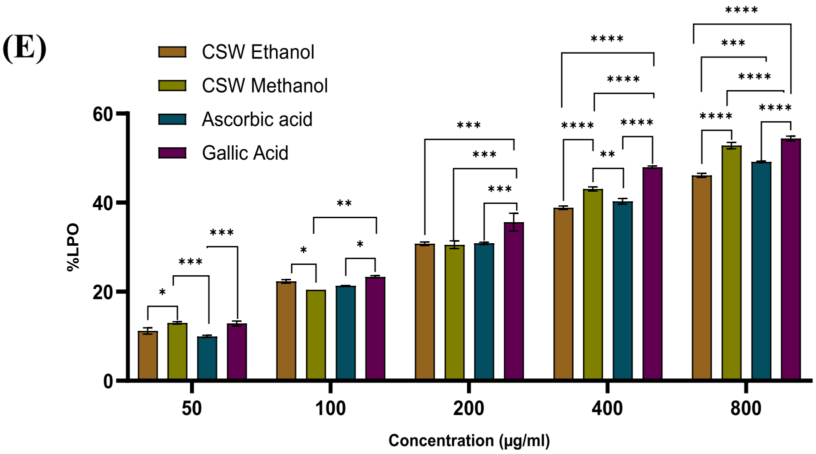

**Fig. 3** (continued)

hydro-ethanol extracts demonstrated significant radical neutralisation potential when compared to standard antioxidants (ascorbic acid and gallic acid controls). The methanolic extract exhibited strong activity, showing concentration-dependent scavenging effects comparable to those of the reference compounds at higher concentrations ($p < 0.05$).

## 4 Discussions

The vaporisation of organic waste, particularly in the form of anaerobically digested slurry, is currently emerging as a promising domain for not only waste management and energy recovery but also for the search for bioactive compounds with potential applications in the medical, pharmaceutical, cosmetic, and industrial sectors [34, 35]. Interestingly, researchers have identified the uses of organic waste, including agricultural residues and animal waste such as cow dung, rich in various phytochemicals, including phenolic compounds, flavonoids, alkaloids, and terpenoids, which have been widely studied for their antimicrobial, anti-inflammatory, and anti-cancer properties [36–39]. However, this study primarily focused on the pharmaceutical importance of vaporising anaerobic digestate from organic waste.

In the study, a broad spectrum of secondary metabolites, including anthraquinones, phenols, coumarins, flavonoids, saponins, glycosides, xanthoproteins, sterols, phytosterols, tannins, quinones, alkaloids, terpenoids, and Vitamin C were present in both hydro-ethanolic and hydro-methanolic crude extracts of CSW as represented in Table 1.

Anthraquinones, phenolic compounds, and coumarins are renowned for their antioxidant and phytoalexin properties, playing pivotal roles in plant defence mechanisms against pathogens and herbivores [40–42]. Flavonoids, serving as pigments, exhibit potent antioxidant activities, further contributing to the therapeutic potential of the extracts [40]. Saponins and glycosides, with their detergent-like and growth-regulating properties, respectively, enhance the biological activities of the extracts [43].

In both extracts, primary metabolites, such as carbohydrates and proteins, were confirmed to be present. The consistent presence of these phytochemicals across both solvents accentuates the inherent richness of CSW as a reservoir of bioactive compounds.

The absence of phlorotannins, volatile oils, and resins in the extracts might be linked to the specific composition of feedstock used for the cows prior to AD and the extraction methods. These phytochemicals may not survive or be selectively broken-down during AD. Factors influencing digestibility and phytochemical retention may include the type of feed and digestion conditions [44, 45].

Zhang et al. [46]. highlighted that methanol and ethanol may not be optimal solvents for extracting highly polar or volatile compounds, such as phlorotannins and essential oils. The solubility disparities further explain the absence of these phytochemicals, as phlorotannins are more soluble in highly polar solvents such as dimethyl sulfoxide (DMSO), and volatile oils often require techniques like steam distillation for effective extraction [47].

The results of the quantitative phytochemical analysis (Fig. 1) demonstrated that both ethanol and methanol are effective solvents for extracting bioactive compounds from CSW, with ethanol showing superior efficiency for total phenolic and flavonoid compounds. Phenolics and flavonoids are well-recognised for their antioxidant, anti-inflammatory, and chemo-preventive properties [73], and their relatively high abundance in the extracts emphasises the therapeutic potential of CSW. The observed superiority of ethanol over methanol in extracting these classes of compounds is consistent with previous reports, which attribute the higher polarity and protic nature of ethanol to its enhancedsolubilisation of polyphenolic structures.

Conversely, other secondary metabolite classes, including saponins, tannins, flavanols, and proanthocyanidins, were detected at low concentrations and did not differ significantly between the two solvents. This suggests that these compounds are either present at inherently low levels in CSW or are equally extractable by both solvents under the conditions employed.

FT-IR spectroscopy (Tables 2 and 3; Fig. 2A, B) provided a molecular fingerprint of the functional groups in crude hydro-methanolic and crude hydro-ethanolic extracts. The identification of functional groups such as hydroxyl (O-H), amide (N-H), carbonyl (C = O), alkene (C = C), and aromatic groups is crucial in understanding the antioxidant activities of crude hydro-extracts. These groups contribute to the antioxidant properties through various mechanisms, including hydrogen atom transfer, electron transfer, and radical adduct formation [57, 58]. The presence and position of these groups can significantly influence the antioxidant efficacy of compounds, as they determine the ability to donate hydrogen atoms or electrons to neutralise free radicals.

A broad absorption band around 3450 $cm^{-1}$, observed in both extracts, indicates O–H stretching vibrations characteristic of free and bound phenolic groups. Phenolic compounds are well-documented for their role as potent antioxidants, capable of scavenging reactive oxygen species (ROS) and mitigating oxidative damage [59, 60]. Previous studies on plant extracts, such as those from *Moringa oleifera* and *Cassia fistula*, reported broad O–H bands between 3400 and 3500 $cm^{-1}$, correlating with high phenolic content and antioxidant potential [65].

The strong C–H stretching bands, detected between 2960 and 2900 $cm^{-1}$ in both extracts, indicate the presence of aliphatic chains, suggesting the presence of saturated hydrocarbons. These structures are commonly associated with membrane-stabilising effects, potentially enhancing cellular resilience against oxidative insults. Comparable C–H stretching features were previously reported in FTIR spectra of cassava peel extracts subjected to biotransformation, confirming the persistence of aliphatic structures post-fermentation [15].

Distinct and sharp C = O stretching vibrations at approximately 1777 $cm^{-1}$ and 1670 $cm^{-1}$ further substantiate the presence of carbonyl-containing functional groups, such as esters, ketones, and amides. These carbonyl groups play essential roles in redox reactions and radical scavenging activities. Studies revealed a similar observation in the FTIR profiles of phenolic-rich plant extracts, including *Terminalia catappa* and *Azadirachta indica*, with strong carbonyl bands aligning closely with their substantial antioxidant activities [61, 66]. Moreover, the C = C stretching vibration detected near 1620 $cm^{-1}$ and the C–O stretching bands between 1260 and 1050 $cm^{-1}$ signify the existence of aromatic and polyphenolic structures within the extracts. Polyphenols, particularly flavonoids and tannins, contribute significantly to the radical scavenging abilities of plant-based extracts by stabilising unpaired electrons and preventing oxidative chain reactions [67]. The presence of aromatic C–H out-of-plane bending vibrations around 750 $cm^{-1}$ further supports the occurrence of substituted aromatic compounds, a feature similarly reported in FTIR analyses of antioxidant-rich extracts from *Camellia sinensis* (tea leaves) [68]. When comparing both extracts, the methanolic extract exhibited relatively stronger absorption intensities for the O–H and C = O functional groups, suggesting a higher concentration of phenolic and carbonyl compounds compared to the ethanolic extract. This trend mirrors findings from several phytochemical investigations where methanol extraction proved more effective at solubilising polar bioactive molecules, such as phenolic acids and flavonoids, thereby enhancing antioxidant activities [69].

NMR was used to target the bioactive and chemical categories of compounds in the hydro-crude extracts [62]. The high signals observed in the NMR spectrum of hydro-crude extracts, particularly from the glutamic acid group (as shown in Tables 4 and 5), suggest potential antibacterial properties targeting bacterial cell walls. This observation aligns with studies indicating that components like poly-γ-glutamic acid (γ-PGA), a derivative of glutamic acid, exhibit biological activities relevant to antibacterial functions [63]. Other compounds, such as harmine, salicylic acid, anacardic acid, cardol, asparagine, gallic acid, quercetin, cardanol, p-cymene, tyrosine, d-germacrene, and alanine, present in the extracts, characterised by diverse functional groups, exhibit complementary biological activities, including antioxidant, anti-inflammatory, and neuroprotective effects [64]. Their functional interaction provides a basis for pharmaceutical exploration, particularly in combating oxidative stress, infections, and chronic inflammatory conditions.

Furthermore, the antioxidant potential of CSW extracts was evaluated through multiple assays, each targeting different reactive species, thereby providing a comprehensive view of the extracts' antioxidant capabilities. The Nitric Oxide (NO) radical scavenging assay of the extracts, as shown in Fig. 3(A), demonstrated that gallic acid exhibited the highest scavenging activity across all concentrations, significantly outperforming both CSW hydro-ethanol and hydro-methanol extracts. Notably, at lower concentrations (50 µg/mL), CSW methanol showed slightly higher activity than CSW ethanol ($p = 0.0195$), suggesting a more effective extraction of NO-inhibitory compounds. However, as the concentration increased, the efficacy of CSW methanol, although superior to CSW ethanol, remained inferior to that of gallic acid [48]. These findings are consistent with the neutralisation of phenolic and flavonoid compounds, which effectively neutralise reactive nitrogen species [49, 50].

In the hydroxyl radical scavenging assay (Fig. 3B), gallic acid again emerged as the most potent scavenger, followed by ascorbic acid and the CSW hydro-methanol extract. The hydro-methanolic extract exhibited a higher scavenging efficacy ($IC_{50} = 530.60$ µg/mL) than the ethanolic extract, aligning with its higher phenolic and flavonoid content. Both extracts showed reduced scavenging activity at concentrations above 800 µg/mL, indicating potential pro-oxidant effects or complex interactions among phytochemicals at higher doses [51].

This attenuation at higher concentrations reflects the dual role of phenolic compounds, which can act as antioxidants at lower concentrations but may exhibit pro-oxidant behaviour under certain conditions [52]. The enhanced activity of the hydro-methanolic extract at lower concentrations reinforces the importance of solvent selection in optimising antioxidant potential.

The hydrogen peroxide ($H_2O_2$) scavenging assay (Fig. 3C) revealed that both CSW methanol and ethanol extracts exhibited significant $H_2O_2$ decomposition activity, with hydro-methanol extract demonstrating superior efficacy ($IC_{50} = 549.71$ µg/mL) compared to the hydro-ethanolic extract ($IC_{50} = 632.45$ µg/mL). Notably, both extracts outperformed ascorbic acid in this assay, highlighting their robust antioxidant capabilities [53].

The efficient scavenging of $H_2O_2$ by the extracts is likely due to the presence of carboxyl and hydroxyl functional groups identified in the FT-IR analysis, which facilitate the neutralisation of reactive oxygen species (ROS). The hydro-methanolic extract's higher phenolic content further enhances its ability to decompose hydrogen peroxide, a critical factor in mitigating oxidative stress.

The DPPH radical scavenging assay [Figure (D)] highlighted that the ethanolic extract exhibited the highest activity among the CSW extracts ($IC_{50} = 513.72$ µg/mL), surpassing the hydro-methanolic extract ($IC_{50} = 662.65$ µg/mL) and even ascorbic acid ($IC_{50} = 695.71$ µg/mL). Gallic acid remained the most potent scavenger ($IC_{50} = 513.72$ µg/mL), aligning with its established efficacy in neutralising stable freeradicals [54].

The superior performance of the ethanolic extract in the DPPH assay, despite lower overall phenolic content compared to the methanolic extract, suggests that ethanol may extract specific DPPH-reactive compounds more efficiently. This discrepancy highlights the nuanced influence of solvent polarity and selectivity on the antioxidant profiles of plant extracts.

In the lipid peroxidation (LPO) inhibition assay (Fig. 3E), the hydro-methanolic extract demonstrated significantly more vigorous inhibitory activity ($IC_{50} = 668.37$ µg/ml) compared to the ethanolic extract ($IC_{50} = 806.40$ µg/ml). Gallic acid consistently exhibited the highest LPO inhibition across all concentrations, reinforcing its role as a potent antioxidant [55, 56].

The hydro-methanolic extract's superior LPO inhibition indicates its higher content of phenolic and flavonoid compounds, which effectively prevent lipid oxidative degradation. The concentration-dependent inhibitory effects emphasise the critical role of dosage in maximising antioxidant efficacy, as higher concentrations did not proportionally enhance inhibition, possibly due to saturation or inhibitory interactions among phytochemicals.

## 5 Conclusion

This study demonstrates that anaerobically digested CSW represents an underutilised source of bioactive compounds, addressing the critical need for sustainable valorisation of organic waste. Our findings establish ethanol as the more suitable solvent for maximising the recovery of phenolic and flavonoid compounds from CSW. This may translate to improved bioactivity profiles in subsequent pharmacological evaluations. The data also provide a foundational understanding of the phytochemical profile of CSW extracts and their characterisation using spectroscopy, which confirms the presence of diverse functional groups and metabolites (e.g., harmine, quercetin), supporting their further exploration as potential sources of therapeutic agents. We recommend that future work on these extracts must prioritise (1) isolation of individual active compounds (e.g., anacardic acid, cardol) to delineate structure-activity relationships and compound-specific bioactivity validation and scale-up optimisation, (2) in vivo efficacy and safety studies, and (3) techno-economic analysis for industrial adoption. Further, this research opens new avenues of microbial bioconversion to transform agricultural waste into waste valorisation and natural product discovery.

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**Author contributions**
ASB and OOP conceptualised and designed the study, with ASB and OOI providing supervision. ASB, ASH, ANB, OOA, and OOP established the research methodology. OOP and AAA conducted the study, while OOI, ASB, ASH, ANB, and OOA performed validation. The original manuscript draft was prepared by OOP and AAA, with ASB and ASH responsible for reviewing and editing the draft. All authors reviewed and approved the final version of the manuscript for submission.

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**Data availability**
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate**
All ethical guidelines have been adhered. There are no studies by any of the authors in this article that used human subjects.

**Consent for publication**
All authors have reviewed and approved the final version of this manuscript and consent to its publication in Discover Chemistry.

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The authors declare no competing interests.

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