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Profiling of Secondary Metabolites from *Crassocephalum crepidioides* (Wild Leafy Vegetable): Validation of Ethnomedicinal Claim by *in-vitro* and *in-silico* Studies

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ABSTRACT: *Crassocephalum crepioides*, labeled as poor man's food is one of the neglected and underutilized vegetables in Nigeria. The objective of this paper is therefore to validate the ethnomedicinal claim and profile the secondary metabolites of *Crassocephalum crepidioides* (Wild Leafy Vegetable) using *in-vitro* and *in-silico* investigations. The identification of compounds in the leaf extract with the potential to inhibit 1Z32 alpha-amylase was carried out *in-silico*. Liquid Chromatography Mass Spectrometry (LCMS) was employed for analysis of the ethanolic leaf extract of this plant, it revealed the presence of sinapic acid, 3-Feruloylquinic acid, dihydroquercetin, malic acid, gallic acid in the extract. Site-directed multi-ligand docking of the identified compounds was performed on 1Z32 protein of alpha-amylase molecular target using the synthetic co-crystallized ligand from the protein. The binding affinity of 3-Feruloylquinic acid (-9.4 kcal/mol) is significantly the highest when compared with sinapic acid (-8.3 kcal/mol). The interactions of this molecule with the amino acids of the protein showed that the mechanism of its inhibitory action is similar to that of the co-crystallized ligand. This study validated an earlier report that the ethanol leaf extract from *C. crepidioides* showed excellent antidiabetic activity, however, the antidiabetic activity could occur through its alpha-amylase inhibitory activity. Data obtained revealed that *C. crepidioides* could be an important wild vegetable that could require further advanced exploration rather than neglect.

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Diabetes, a metabolic disorder, is characterized by aberrant carbohydrate, protein, and fat metabolism resulting from insulin deficiency or malfunction, as stated by Junejo *et al*. (2020a). This condition is typically identified by hyperglycemia in conjunction with polyuria, polydipsia and polyphagia. Additional prevailing symptoms include weight loss, fatigue, irritability, blurred vision, slow-healing wounds/sores and reduced susceptibility to infections (Junejo et al., 2020b). Furthermore, persistent hyperglycemia may give rise to severe complications such as diabetic cardiomyopathy, neuropathy, nephropathy and retinopathy (Junejo *et al.*, 2020c). In light of the global upsurge of lifestyle disorders and associated cardiovascular diseases such as obesity, heart diseases and hyperlipidemia, diabetes is increasingly posing a significant threat to public health (Sunil et al., 2021). As per recent reports, diabetes affects approximately 3.0% of the global population and is expected to rise to 4.4% by 2030 (Junejo et al., 2020b). Type 2 diabetes, which is also known as non-insulin-dependent diabetes mellitus, contributes to 90% of the overall prevalence of diabetes, making it the fourth or fifth leading cause of death worldwide according to Li et al. (2019) report. Phytotherapeutic agents derived from herbs and shrubs have been employed as traditional medicine globally since ancient times. Within the West African subcontinent, traditional herbal remedies constitute an essential component of medicines, with historical roots (Mukherjee et al.,

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2018). The management of diabetes mellitus is significantly influenced by plant-based drugs and/or herbal formulations (Day, 1998). The World Health Organization (WHO) advocates for the assessment of traditional herbal medicines to confirm their therapeutic efficacy against diabetes (Rupeshkumar et al., 2014; Junejo et al., 2020a), as they are cost-effective and safe compared to modern medicines. Presently available antidiabetic interventions, encompassing synthetic oral hypoglycemic agents such as sulfonylureas, biguanides, meglitinides, glitazones, and gliptins, have been linked to a range of severe life-threatening side effects like anorexia, drowsiness, weight gain, and abdominal discomfort (Majumdar and Inzucchi, 2013; Barky et al., 2016). In this regard, a comprehensive scientific examination of traditionally valuable medicinal plants (inclusive of herbal medicines) is imperative to explore their antidiabetic potential with the aid of modern experimental tools and techniques. Structure-based virtual screening of natural product databases as well as target fishing approaches have experienced increased utilization in the field of natural product research, allowing for the systematic identification of promising compounds and novel molecular targets for bioactive compounds (Junejoa et al., 2021). It facilitates the detection of fresh medications from natural products, utilizing the *in silico* drug design methodologies. Currently, the field of natural product research has observed an increase in the utilization of structure-based virtual screening of natural product databases, along with target fishing approaches ((Junejoa et al., 2020a). These methodologies allow for the systematic identification of promising compounds, while simultaneously exploring novel molecular targets for bioactive compounds. The Asteraceae family of medicinal plants can be found in diverse ecological habitats globally and has a rich history in traditional medicine. The Crassocephalum genus encompasses 24 acknowledged species, among which *Crassocephalum crepidioides* is included, commonly known as 'fireweed ragleaf' in English, 'ebolo' in Yoruba, 'Obuinenawa' in Edo tribe (South West of Nigeria) and 'Anikale jhar' in Nepali. The plant has numerous traditional uses, substantiated by scientific studies. In Africa, *C. crepidioides* is consumed as leafy vegetables (Owokotomo and Owokotomo, 2018). Pharmacological activities, such as antibacterial, wound healing, antidiabetic, anti-inflammatory, and antioxidant properties, have been attributed to the plant (Adebayo *et al.,* 2015; Akinpelu *et al.,* 2019; Tomimori *et al.*, 2012; Bahar *et al.,* 2017; Aniya *et al.,* 2005). Therefore, the objective of this paper is to validate the ethnomedicinal claim and profile the secondary metabolites of *Crassocephalum crepidioides* (Wild Leafy Vegetable) using *in-vitro* and *in-silico* investigations.

MATERIAL AND METHODS

Collection, Identification and Drying of the Plant: The leaves of *Crassocephalum crepioides* were collected in November 2021, at Ilorin, Kwara State, Nigeria. It was identified at the Department of Life Sciences at the Federal University of Dutsin-Ma, Katsina. In the laboratory, the leaves were cleansed with water and air-dried for two weeks. They were ground with a pestle and mortar. The powdered samples were maintained at room temperature in clean, airtight containers until they were needed.

Extraction and Concentration: Ethanol was used to extract the powdered leaf sample. Ethanol was utilized as the extraction solvent for 7 days on 1 kg of powdered leaf sample packed in Bama bottles. The solvent was collected by a rotary evaporator at the end of the period. The extract was fractionated using a separation funnel and Hexane as the solvent, the polar and the non-polar fraction were collected. After that, the extracts were placed in a desiccator and allowed to dry fully before being tested.

Alpha amylase inhibitory activity: The ethanol extract of the leaves of *C. crepidioides* was subjected to the α-Amylase inhibitory ability of the extract was decided, employing a reported method though by Zhang et al. (2011).

Liquid Chromatography-Mass Spectrometry (LCMS): Protocol for LCMS Analysis (Generic Method) using LC Waters's e2695 separation module with W2998 PDA and couple to ACQ-QDA MS. The ethanol extract of the leaves of *C. crepidioides* was analyzed using liquid chromatography (LC) tandem mass spectrophotometer (MS) as described by (Piovesana et al., 2018) with some modifications. The extracted samples were reconstituted in methanol and filtered through a polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. After filtration, the filtrate (10.0 µl) was injected into the LC system and allowed to separate on Sunfire C18 5.0 µm 4.6 mm x 150 mm column.

Table 1: Solvent System for the LCMS

Time	% A	% B
0	95	5
1	95	5
13	5	95
15	5	95
17	95	5
19	95	5
20	95	5

The run was carried out at a flow rate of 1.0 mL/min, with Sample and Column temperature at 25ºC. The

mobile phase consists of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a gradient as in Table 1.

The ratio of A/B 95:5 this ratio was maintained for a further 1 min, then A/B 5:95 for 13 min, to 15 min. then A/B 95:5 to 17 min, 19 min and finally 20 min. the PDA detector was set at 210-400 nm with a resolution of 1.2 nm and a sampling rate of 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8 kv (positive) and 0.8 kv (negative); probe temperature 600oC; flow rate 10 mL/min; nebulizer gas, 45 psi. MS is set in automatic mode applying a fragmentation voltage of 125 V (Piovesana et al., 2018). The data was processed with Empower 3. The compounds were identified based on the following information, elution order, and retention time (Rt), fragmentation pattern, and Base m/z.

Identification and preparation of molecular target: The molecular target protein human pancreatic alpha-amylase (PDB ID: 1Z32) (Fig. 2) was identified from literature ((Nahoum et al., 2000) and downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org/). The crystallographic water molecules and cocrystallized ligands that were interfering were eliminated, and the protein's energy was reduced through the utilization of UCSF Chimera 1.14. The minimization process involved 300 steepest descent steps at 0.02 Å. Ten conjugate gradient steps were conducted at 0.02 Å and ten update intervals. To ensure a suitable structure conformation, Gasteiger charges were integrated using Dock Prep, as suggested by Duru *et al.* (2021a).

Ligand Identification and preparation: The 3D structure-data files (SDF) of the compounds identified in the ethanol extract of the leaves of *C. crepidioides* from the LC-MS analysis were selected and downloaded from the PubChem database. They were minimized in PyRx virtual screening tool, using Universal Force Field at 200 steps, then converted to AutoDock ligands (pdbqt) and used for the docking analysis.

Docking procedure and analysis of results: The compounds identified from the ethanol extract of the leaves of *C. crepidioides* underwent screening on the enzyme pocket that bears the cocrystallized ligand EEK. The amino acids located at this binding site were identified through the utilization of UCSF Chimera 1.14 software, and the site underwent validation using PyMOL software. The multiple docking of the ligands on the enzyme was carried out with Autodock Vina in PyRx software (Tsao et al., 2020; Duru et al., 2020), with center grid box sizes of x center: 9.7977, y center: 42.5668, and z center: 19.5188 and the dimension x centre: 31:0360, y centre: 28.5115, and z centre: 23.7148 The binding affinities of the compounds on the protein target were acquired after docking, and the outcomes were systematically organized on an Excel spreadsheet.

Analysis of protein-ligand interactions: The examination of protein-ligand interactions in the optimal compounds of the extract was carried out by analyzing the distinct amino acid residues of the protein using Biovia Discovery studio client 20.1 (BIOVIA, 2020).

RESULTS AND DISCUSSION

Chromatography serves as an analytical instrument for the separation and quantification of mixture components.

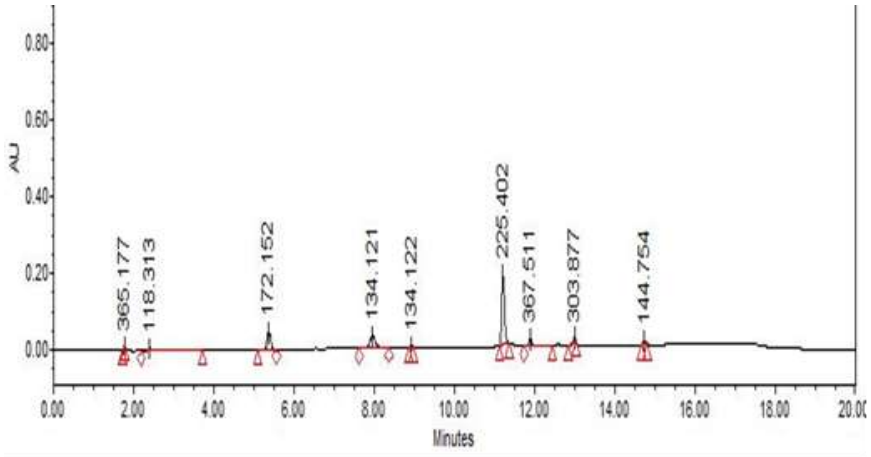

Fig 1: Chromatogram of Secondary Metabolites in *C. crepidioides*

Table 2: Compounds identified from Figure 1

S/N	Identified compounds	Molecular formula	Calculated mass	Precursor ion, m/z [M-H]⁻	[M+H]⁺	Fragmentation
1	Sinapic acid	$C_{11}H_{12}O_5$	225.402	224.212		**164**, 149, 208, 164, 193, 179
2	3-Feruloylquinic acid	$C_{17}H_{20}O_9$	**367.511**	367.1034		298, 288, 192, 191
3	Dihydroquercetin	$C_{15}H_{10}O_7$	303.877	303.0508		285; 163; 267; 159; 239
4	Malic acid	$C_4H_6O_5$	134.122	133		115
4	Malic acid	$C_4H_6O_5$	134.121	133		115
5	Hexose-hexose-Nacetyl	$C_{14}H_{25}NO_{10}$	365.177	366		186; 142
6	Gallic acid	$C_7H_6O_5$	172.152	171		126

The outcome of chromatography, as indicated by a chromatogram, presents the separated components of the mixture. The distinct peaks observed on the chromatogram are indicative of the compounds found within the analyzed crude extract. The numerical values assigned to the peaks correspond to the elution times of the compounds during the chromatographic process. The Liquid chromatogram for the phytochemicals present in the ethanolic leaf extract of *C. crepidioides* exhibited 6 peaks, which are exhibited in Figure 1. The identified pytocompounds, their compound identification number on the PubChem database, and their structures are summarized in Table 2 (Patel and Narasimhacharya, 2022, Mishra et al., 2022, Duru and Duru, 2019). The classes in which these compounds fall include Hydroxycinnamic acid: compounds that fall including this category is 3-feruloyl quinic acid (**2**) and sinapic acid (**1**) Hydroxybenzoic acid which includes gallic acid (**6)** Flavones: Dihydroquercetin (**3**). Other compounds include malic acid (**4**).

Biological Activity of the Leaves extracts of C. crepioides: Table 3 shows that the inhibition activity of both plants is dependent on the dosage. The leaf extracts of *C. crepioides* showed an increase in activity with an increase in dosage (500 µg/ml). At 50 µg/ml dose, the activity of *C. crepioides* was found to be better than that of the antidiabetic drug acarbose. At lower concentrations of 20 µg/ml and 50 µg/ml, the inhibitory influence of the ethanolic extract of *Crassophalum crepidioides* on alpha-amylase activity is comparatively lower than that of acarbose.

Table 3: α-amylase activity of the Leaves extracts of *C. crepioides*

Dose	*C. crepioides*	Acarbose
20 µg/ml	45.8169935	42.4836601
50 µg/ml	56.4705882	58.0065359
100 µg/ml	67.2875817	63.8888889
250 µg/ml	77.9411765	67.1568627
500 µg/ml	81.6993464	68.627451

Conversely, as the concentration of the ethanolic extract of *C. crepidioides* increases, the inhibitory impact on alpha-amylase activity also ascends. The inhibitory activity of the extract of *C. crepidioides* shows a gradual increase with higher concentrations of the extract. At more substantial concentrations (250 µg/ml and 500 µg/ml), the inhibitory effect of the ethanolic extract of *C. crepidioides* becomes comparable to or even slightly greater than that of acarbose. These observations intimate that the ethanolic extract of *Crassphalum crepidioides* has an inhibitory effect on alpha-amylase activity, and the extent of inhibition amplifies with higher concentrations of the extract. At higher concentrations, the inhibitory effect of the extract becomes comparable to that of acarbose, which is a well-known inhibitor of alpha-amylase. This suggests that wild vegetable show be taken more for effective health-promoting abilities to be obvious.

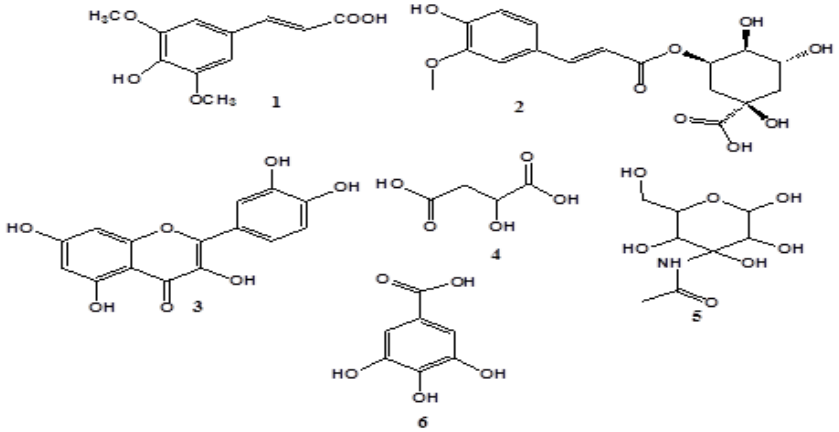

Fig 3: Secondary Metabolites identified in *C. crepidioides*

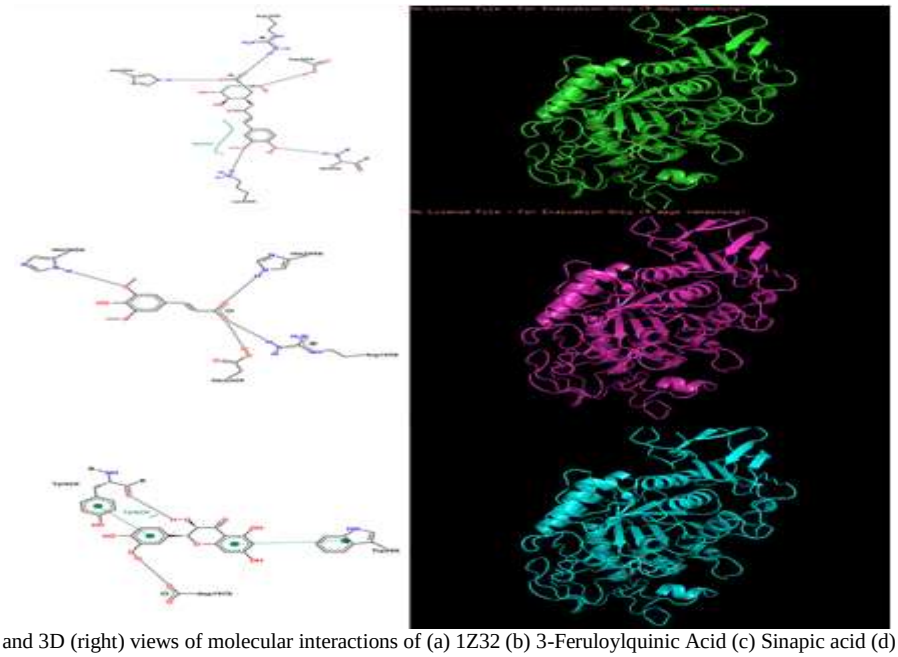

Fig 4: 2D (left) and 3D (right) views of molecular interactions of (a) 1Z32 (b) 3-Feruloylquinic Acid (c) Sinapic acid (d) Dihyroquercetin

The binding affinities of the compounds in the leaf extract of *C. crepidioides* on human pancreatic alpha-amylase (PDB ID: 1Z32) are shown in Table 3. From protein-ligand interactions, favourable binding modes or/and orientations of Hydroxycinnamic acids and flavonoid compounds identified from the wild vegetable, for alpha-amylase enzyme were evident. Table 5 shows the details of protein-ligand interactions illustrating the related amino acid residues and various types of interactions involved. The best ligand with the binding affinity (ΔG (kcal/mol)) of -9.2 is 3-Feruloylquinic Acid with the interacting residues: Ile235, Lys200, Asp200, His299, Arg195, Ile235 with more hydrophobic and electrostatic energies but displayed various interactions: Pi-sigma, Van der Waals, Van der Waals, Van der Waals, Van der Waals, Pi-sigma. The interaction between 3-Feruloylquinic acid and alpha-amylase protein entails a confluence of hydrophobic and electrostatic interactions. The hydrophobic interactions are presumed to occur between the ligand and hydrophobic residues, particularly Ile235 and Ile235, which contribute to the stabilization of the complex. The electrostatic interactions, comprising of Van der Waals forces and Pi-sigma interactions, encompass charged residues (Lys200, Asp200, His299, and Arg195) that potentially establish bonds with the ligand. These interactions are likely to contribute to the inhibitory effect of 3-Feruloylquinic acid on the activity of the alpha-amylase enzyme (Duru et al., 2021a; Duru et al., 2021b). The binding of Sinapic acid to alpha-amylase protein, possessing a binding affinity of -8.3 kcal/mol, predominantly involves electrostatic interactions and hydrogen bonding. The Van der Waals forces signify the electrostatic interactions between the ligand and residues Arg195 and Glu233. Furthermore, the ligand demonstrates the formation of hydrogen bonds with His200 and His305, perhaps by engaging with the functional groups that are present in Sinapic acid. These interactions likely account for the inhibitory impact of Sinapic acid on the alpha-amylase enzyme (Duru et al., 2020). The binding affinity of Dihydroquercetin, a flavonoid, exhibits a lesser value (-6.2 kcal/mol) as compared to the top three binders in its interaction with the alpha-amylase protein. This interaction is a conglomeration of hydrophobic, electrostatic, and hydrogen bonding interactions.

Table 4: Binding affinities of compounds in *C. crepidioides* leaf extract on human pancreatic alpha-amylase (PDB ID: 1Z32)

S/N	Name	Mol. Wt g/Mol	PUBCHEM ID	CANONICAL SMILES	Binding Affinity ΔG (kcal/mol)
1	Sinapic Acid	224.21	637775	COC1=CC(=CC(=C1O)OC)C=CC(=O)O	-8.3
2	3-Feruloylquinic Acid	368.3	10133609	COC1=C(C=CC(=C1)C=CC(=O)OC2CC(CC(C2O)O)(C(=O)O)O)O	-9.3
3	Dihyroquercetin	304.25	439533	C1=CC(=C(C=C1C2C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O	-6.2
4	Malic Acid	134.09	525	C(C(C(=O)O)O)C(=O)O	-4.8
5	Gallic Acid	170.12	370	C1=C(C=C(C(=C1O)O)O)C(=O)O	-5.9

Table 5: Details of the best three protein-ligand interactions with protein 1Z32

	Ligand	Interacting residues	Category	Type of Interaction
1	3-Feruloylquinic Acid	Ile235, Lys200, Asp200, His299, Arg195, Ile235	Hydrophobic Electrostatic Electrostatic Electrostatic Electrostatic hydrophobic	Pi-sigma Van der waal Van der waal Van der waal Van der waal Pi-sigma
2	Sinapic Acid	Arg195, Glu233, His200, His305	Electrostatic Electrostatic H-bond H-bond	Van der waal Van der waal Pi-pi stacked conventional
3	Dihyroquercetin	Try62, Tyr62, Asp197, Trp59	Electrostatic Hydrophobic H-bond Electrostatic	Van der waal Pi-sigma Conventional Van der waal

The ligand is seen to engage with Try62 and Tyr62 residues through hydrophobic interactions and Van der Waals forces. Additionally, it forms a hydrogen bond with Asp197 and an electrostatic interaction with Trp59 (Mukherjee et al., 2018 & Duru and Duru, 2019). These interactions potentially contribute to the inhibitory impact of Dihydroquercetin on the alpha-amylase enzyme. Hydroxycinnamic acids and polyphenols are distinguished by the presence of a carbon-to-carbon double bond that is conjugated with a carbonyl group within their structural makeup. This characteristic feature serves to stabilize the binding forces to the active site of the α-amylase (Giubert et al., 2020). Furthermore, the significance of hydroxyl groups of polyphenols on the interaction with amino acid residues at the active site of α-amylase (Glu233) has been widely acknowledged (Sun et al., 2019). Sun et al. (2019) discovered and suggested through molecular modeling that the removal of hydroxyl groups of Hydroxycinnamic acids and polyphenols may decrease the inhibition effect. Molecular docking analysis has suggested that the elimination of hydroxyl groups of polyphenols might potentially reduce the inhibition effect (Sun *et al.,* 2019).

Conclusion: The identification of human pancreatic alpha-amylase (PDB ID: 1Z32) inhibiting compounds in ethanol leaf extract of *Crassophalum crepidioides* was carried out using *in silico* methods. The molecule 3-Feruloylquinic acid gave the highest binding affinity on the alpha-amylase 1Z32 enzyme relative followed by Sinapic acid as to the other components in the extract. These compounds are substantially present in the leaf of this plant. This neglected and underutilized vegetable is mostly eaten by people in rural places, its functional abilities revealed by this study should be harnessed. The assertion that "there is no or little research on these wild leafy vegetables" is changing, this study is reducing the narrative. This study shows the importance of *C. crepidioides* and gives evidence why this wild vegetable needs to be explored more and not neglected.

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