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SECONDARY METABOLITES PROFILING USING LC-MS AND ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACTS OF THE LEAVES *Crassocephalum crepioides*

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ABSTRACT

Harnessing the vast supply of underutilized crops would provide several opportunities to increase the temporal and geographical variation in cropping systems, which would ultimately result in a more sustainable supply of a variety of healthy foods for everyday consumption. *Crassocephalum crepioides* is one of neglected and underutilized vegetables in Nigeria. *C. crepioides* is a vegetable labelled as poor-man's food because its medicinal importance and phytocompounds profiling is yet to be ascertained. They reputed to be employed in managing variety of ailments such as sores, chest pains, diarrhea, and menstrual cramps, diabetes, inflammation, enteritis. Liquid Chromatography Mass Spectrometry (LCMS) was used to identify the compounds presents in the ethanol extract of the leaves of *C. crepioides*. The antidiabetic activity of the plant was evaluated employing α-amylase assay while Hydrogen peroxide (H_2O_2), ABTS and DPPH inhibition assays were employed for antioxidant activity. LCMS shows six (6) compounds of different chemical classes and of diverse biological application were identified in the plant extract base on their m/z ratio i.e. hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, alkaloids and other benzoic acids. The ABTS inhibition assay gave the best result with 16.21% at 20 ug/ml though the extract is dose dependent as the activity decreases with corresponding increase in the dosage. This vegetable should not be neglected as the study reveals, *C. crepioides* contain natural products useful against diseases and ailments and consumed in large quantities in order to have good effects against problems related to oxidative stress.

Keywords: *C. crepioides*, α-amylase, neglected and underutilized vegetable, LC-MS

INTRODUCTION

By utilizing underutilized crops, temporal and spatial heterogeneity can be introduced into cropping systems, which ultimately leads to a more sustainable supply of diverse and nutritious food for routine consumption (Padulosi et al., 2002). This approach can ultimately lead to a more sustainable supply of diverse and nutritious food for routine consumption. Underutilized crops, previously confined to specific regions, offer a viable means of meeting the food and nutritional needs of a rapidly growing human population. Notably, NUS legumes have an excellent nutritional profile and represent an affordable alternative source of protein, critical for sustaining livelihoods and genetic resources in the face of unpredictable and hostile climatic conditions (Kahane et al., 2013). The Sustainable Development Goal of 'Zero Hunger' established by the United Nations poses a significant challenge in numerous countries, as hunger and malnutrition persist as rampant issues (Li and Siddique, 2020). Global food and nutritional insecurity is significantly contributed by factors such as limited available cropping land, over-reliance on a few staple crops, volatile prices of nutritionally wholesome food, changing climatic conditions, and the emergence of pandemic diseases (Katoch, 2020).

Crassocephalum crepidioides is one of the neglected and underutilized plant species in West Africa countries. These plant species are sometimes called wild leafy vegetables, these vegetables are often overlooked by individuals in developed nations, serve as a means of subsistence agriculture in developing countries, particularly within regions facing food insecurity. Inhabitants of remote areas possess extensive expertise in the utilization of these wild species as sustenance, particularly during periods of drought, famine, and civil unrest. The understanding of these wild species may be considered the most significant factor in determining an individual or family's ability to maintain nutritional well-being, fall victim to malnourishment, or succumb. *Crassocephalum crepidioides* known by various English names such as thickhead, fireweed, Okinawa spinach, and red flower rag-leaf, is a botanical species that finds its usage in many tropical and subtropical regions (Tomimori et al., 2012). Its prevalence is particularly noteworthy in tropical Africa and the Okinawa Islands in Japan (Burkill, 1995; Tomimori et al., 2012). *C. crepidioides* is widely grown in Asia for its medicinal and nutritional properties, renowned for its effectiveness as a remedy for acute hepatitis and fever (Yoko Aniya et al., 2005). The plant thrives in moist areas, natural grasslands, riverbanks, wastage places, roadside and backyard gardens that are rich in organic matters (Arawande et al., 2013). The Yoruba tribe in South-West Nigeria refers to it as 'Efo Ebolo or Ebire', while the Japanese call it "Benibana borogiku". Studies have reported the effectiveness of *C. crepidioides* in treating a variety of ailments, including indigestion, stomach upset, fresh wounds, headache, and epilepsy (Entaz et al., 2016). The plant has also been found useful in halting nosebleeds and treating sleeping sickness. Tannin, present in the roots of the plant, is particularly effective in treating swollen lips (Adams, 1963). The purpose of this study was ascertain the antidiabetic, antioxidant activities and secondary metabolites profiling of the ethanol extract of the leaves of *C. crepidioides*

MATERIALS AND METHOD

Chemicals and Reagents

All the chemicals and reagents used in the analysis were of analytical grades and obtained from the Department of Applied Chemistry, Federal University Dutsin-Ma, Katsina State. N-hexane, ethanol amongst others was used in the work.

Apparatus and Equipment

This include but not limited to; rotary evaporator, glass bottles, TLC sheets, TLC chambers, water bath, separating funnel, Whatman filter paper, measuring cylinder, hand gloves, masking tape, beakers, spatula, syringes and weighing balance.

Collection, Identification and Drying of the Plant

The leaves of *Crassocephalum crepioide*s 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 a period of 7 days on 1000g of powdered leaf sample packed in Bama bottles. The solvent was collected by rotary evaporator at the end of the period. The extract was fractionated using a separation funnel and Hexane as 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

α-Amylase inhibitory ability of the extract was decided, employing a reported method though by Zhang et al. (2011).

2, 2′-azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity.

The 2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonate, ABTS radical cation decolourization assay based on the scavenging of ABTS•+ radicals by antioxidants component of the extracts was used. The assay follows the procedure of Atolani et al. (2013), with slight modifications (Atolani et al., 2013). All analysis was determined in duplicate.

2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging assay

DPPH is one of the main assays used in assessing the antioxidant activity of plant material employed in this study. The assay was carried out in harmony with the method described by Oguntoye et al. (2018)

Liquid Chromatography Mass Spectroscopy (LC-MS)

The diluted ethanol extract (1 mg/mL) was used for LC-MS/MS analysis. LC-MS triple quadrupole mass spectrometer (Shimadzu 8040) was the main part of the LC-MS/MS system. The ionizations were detected by ESI. The binary pumps (LC-30AD), a column oven (CTO-10ASvp) degaser (DGU-20A3R), and auto sampler (SIL-30AC) were integrated to the LC system. The chromatographic separation was performed on a C18 (150mm x 4.6mm, 3µm) reversed phase analytical column (Inertsil ODS-4). The mobile phase A consisted of 5 mM

ammonium formate, water and 0.1% formic acid. The mobile phase B consist of 5mM ammonium formate 0.1% formic acid and methanol. The injection volume of sample was 4µL. HPLC was run at 0.5mL/min flow. The multiple reaction monitoring (MRM) mode was used to quantify the analyzes. The optimum ESI conditions were set as 350° C for interface temperature, 250° C for DL temperature and 400° C for heat block temperature, 3L/min for nebulizing gas flow and 15L/min for drying gas flow. The analyses of samples were carried out after three transitions for the samples. First transition was for quantitative aim and second and third transitions were for verification.

RESULTS AND DISCUSSION

Result of LC-MS Analysis of Ethanol Extract of *C. Crepioides*

	Retention Time (min)	Scan Number	Name	Baseline Correction	Noise Threshold (%)	Peak Separation (Da)	Max. Intensity (Intensity)	Base Peak (m/z)	Start Mass (m/z)	End Mass (m/z)	Spectra To Average	Baseline Spectra	Acquisition Mode	Display Mode	Smoothing Type	Smoothing Window (Da)	Combine Type	
1	1.762	660		☑	0.000	1.0000	4522.212917	365.177	50.000	600.000	5	10	Centroid				Average	Baselin
2	2.384	894		☑	0.000	1.0000	6761.040527	118.313	50.000	600.000	5	10	Centroid				Average	Baselin
3	5.346	2005		☑	0.000	1.0000	659573.720433	172.152	50.000	600.000	5	10	Centroid				Average	Baselin
4	7.947	2982		☑	0.000	1.0000	444195.580485	134.121	50.000	600.000	5	10	Centroid				Average	Baselin
5	8.921	3347		☑	0.000	1.0000	8464.392170	134.122	50.000	600.000	5	10	Centroid				Average	Baselin
6	11.210	4207		☑	0.000	1.0000	46796.038513	225.402	50.000	600.000	5	10	Centroid				Average	Baselin
7	11.888	4461		☑	0.000	1.0000	209391.274231	367.511	50.000	600.000	5	10	Centroid				Average	Baselin
8	12.994	4876		☑	0.000	1.0000	11860.619743	303.877	50.000	600.000	5	10	Centroid				Average	Baselin
9	14.740	5532		☑	0.000	1.0000	4148.958252	144.754	50.000	600.000	5	10	Centroid				Average	Baselin

Figure 1: m/z ratio of compounds identified against Leaves extracts of *C. Crepioides*

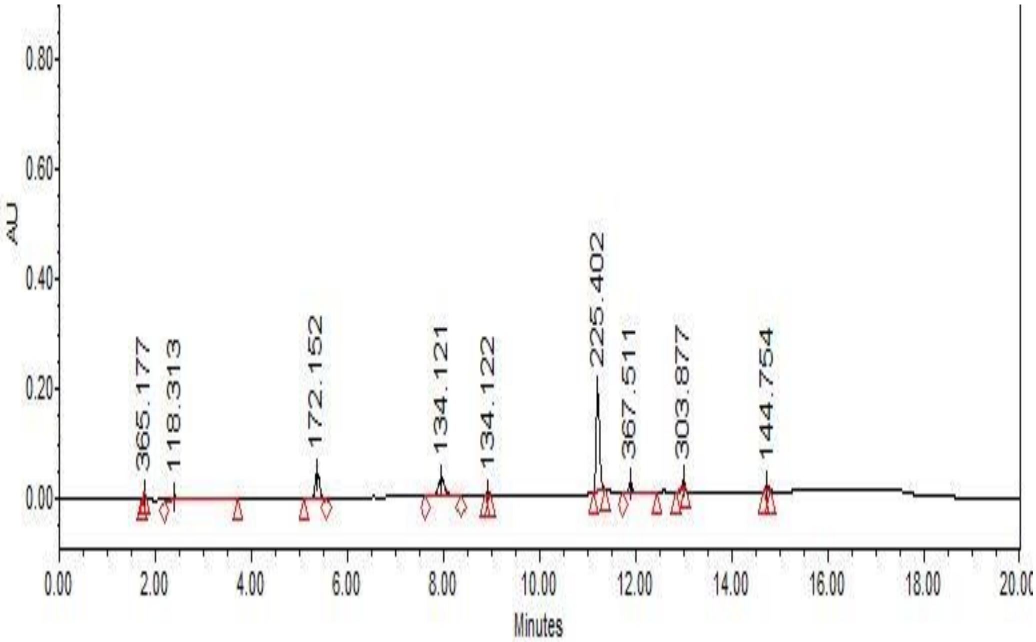


Figure 2: Spectra for *C. crepioides*

Table 1: 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

Biological Activity of the Leaves extracts of *C. crepioides*

Table 2: α-amylase activity of the Leaves extracts of *C. crepioides*

DOSE	*C. crepioides*	ACARBOSE
20 µg/ml	75.8169935	42.4836601
50 µg/ml	76.4705882	58.0065359
100 µg/ml	77.2875817	63.8888889
250 µg/ml	77.9411765	67.1568627
500 µg/ml	81.6993464	68.627451

Table 2 shows that the inhibition activity of both plants is dependent on the dosage. The leaves extracts of *C. crepioides* showed decrease in activity with increase in dosage (500 µg/ml). At 250 µg/ml dose the activity of *C. crepioides* was found to be better than that of the antidiabetic drug acarbose.

Table 3: DPPH Activity of Leaves extract of *C. crespioides*

DOSE	*C. crespioides*	Ascorbic Acid
20 µg/ml	24.71812	30.07525
50 µg/ml	42.57524	41.82336
100 µg/ml	70.67672	51.97373
250 µg/ml	75.65792	60.80831
500 µg/ml	76.12784	65.41357

Table 3 above shows the DPPH inhibition assay of *C. crespioides*. The activity of the extract is dose dependent. The activity of *C. crespioides* decrease with corresponding increase in the concentration of the extract but showed the best activity at minimum dosage. The activity of *C. crespioides* is better than that of the control at all dosages.

Table 4: H_2O_2 activity for Leaves extract of *C. crepioides*

Dose	*C. crepioides*	Ascorbic Acid
20 µg/ml	5.1269	11.8782
50 µg/ml	27.6311	26.6836
100µg/ml	63.0457	39.4755
250µg/ml	69.3232	63.2826
500 µg/ml	69.9154	74.6193

Table 4 above shows the H_2O_2 activity of *C. crepioides.* The table shows that the inhibition activity of *C. crepioides* leaves extract is dose-dependent. There is increase in the activity of both polyphenolic rich extract with decrease in dosage with optimum activity achieved at dosage of 20 µg/ml. At this dose the activity was found to be better than that of the conventional positive control ascorbic acid.

Table 5: ABTS inhibition assay of the Leaves extracts of *C. crespioides*

Dosage	*C. Crepioides*	Ascorbic Acid
20 µg/ml	16.2119	27.39433
50 µg/ml	21.1878	55.80524
100 µg/ml	75.70894	78.22365
250 µg/ml	90.10166	86.35634
500 µg/ml	91.97432	90.10166

Table 5 shows the activity of the leaves extract of *C. crespioides* on ABTS inhibition assay. The result showed that the activity of the extract is dose dependent as the activity decreases with corresponding increase in the dosage. The best inhibition on ABTS (16.21) was recorded at 20 ug/ml which was better than that of the control.

Discussion

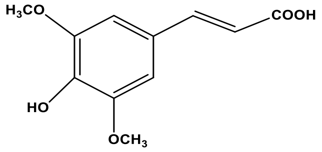
In this current study, a total of six (6) compounds were tentatively identified by comparing the mass to charge m/z of the compounds as perceived by the LC-MS spectrometer and the structures elucidated above. The classes which these compounds fall include **Hydroxycinnamic acid:** compounds that fall 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**).

Hydroxycinnamic Acid

Result interpreted from the LC-MS spectrum shows that 3-feruloyl quinic acid (**2**) and sinapic acid (**1**) fall in the class of hydroxycinnamic compounds or other phenolic acid derivatives. Compound (**2**) was identified as 3-feruloyl quinic acid precursor ($[M - H]^-$ m/z at 367.1038) which was found to be present in both leaves of ***Crassocephalum crepioides*** confirmed by the fragments at m/z 298, m/z 288, m/z 192 and m/z 191, corresponding to the loss of $[M\text{-}H\text{-}3H_2O_2\text{-}CH_3]$, $[M\text{-}H\text{-}H_2O\text{-}CH_3\text{-}HCOOH]$, $[M\text{-}H\text{-}C_7H_{11}O_5]$ and $[M\text{-}H\text{-}C_{10}H_8O_3]$, respectively (Wang *et al.*, 2017).

Compound (**1**) characterized as Sinapic acid with precursor ($[M - H]^-$ m/z at 225) which was found present in the extract of ***Crassocephalum Crepioides*** and confirmed by fragments at m/z 179, m/z 153, and m/z 210 which was corresponded to the loss or cleavage of [M-H- HCOOH], $[M\text{-}H\text{-}CH_2CHCOOH]$ and $[M\text{-}H\text{-}CH_3]$ respectively (Lin *et al.*, 2019).

Hydroxycinnamic acids (HCAs) are important phytochemicals possessing significant biological properties. HCAs are widely distributed across the plant kingdom and are plentiful in whole grains, tea, coffee, red wine, numerous fruits, and vegetables (Sroka and Cisowski, 2003). They have been classified as both bioactive components of food and structural and functional components of plant cell walls. HCAs have a basic phenylpropanoid structure as



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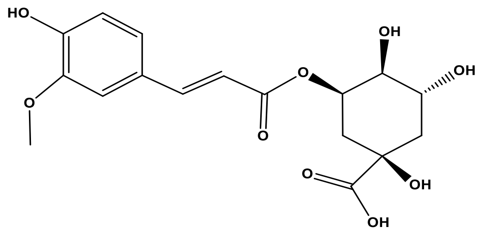

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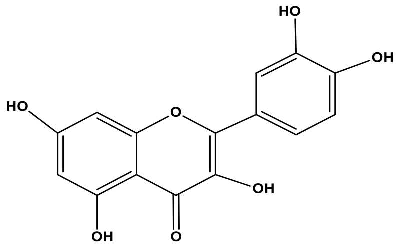

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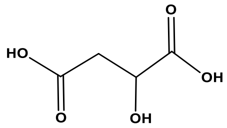

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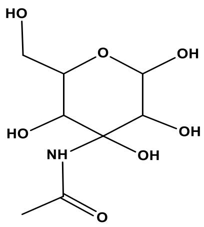

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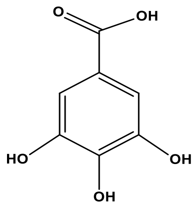

6

as their chemical core. Since quinic acid or the glucose molecule serve as their basic building blocks, natural HCAs often occur as either free forms or esters (Kim *et al.,* 2006). In plants, however, they can also take on more complex derivatives including dimer, trimer, or mixed glycosidic forms. The most noteworthy HCAs are para-coumaric acid, ferulic acid, sinapic acid, and caffeine. Their derivatives have a wide range of biological actions, including antitumoral, antibacterial, antioxidant, and neuroprotective properties.

The phytochemical sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) is widely present in spices, citrus and berry fruits, vegetables, cereals, and oilseed crops. It is known to have antibacterial, anti-cancer, anti-inflammatory, anti-mutagenic, antioxidant, and anticancer properties (Chunye, 2015). The ability of sinapic acid to neutralize the paramagnetic stable radical of 2, 2-diphenyl-1-picrylhydrazyl is another property that it is known to possess (DPPH).

Sinapic acid inhibits DPPH by 33.2% (Sawa *et al.,* 1998), 88.4% (Nenadis and Tsimidou, 2002), and 50% at concentrations of 0.02 mM, 0.5 mM, and 0.3 mM, respectively, according to the literature. Furthermore, at concentrations greater than 200 M, the 8-8-bislactone-dimer of sinapic acid exhibits DPPH scavenging action. These amongst other points could be the reason for the good antioxidant property of the fruit as proposed by ethnomedicinal uses of the plant.

Hydroxybenzoic Acids

one compound is found in this group which is Gallic acid (**6**) with a m/z of 171 with a fragmentation of m/z 126 due to loss of [M-H= CO_2]. Gallic acid is a plant-derived phenolic acid, or bioactive molecule. It contains antioxidant effects and may provide further health advantages. According to some research, gallic acid lowers excessive fat storage in obese people by decreasing lipogenesis. Lipogenesis is the process by which fat is synthesized within the body from substances such as carbohydrates. Gallic acids provides efficient protection against oxidative damage caused by reactive species often encountered in biological systems including, hydroxyl ($HO^{\cdot}$), superoxide ($O_2^{\cdot}$), and peroxyl ($ROO^{\cdot}$) and the non-radicals, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Furthermore, GA has been demonstrated as the chief antioxidant component responsible for the efficient antiradical and anticancer properties of a number of plant extracts. Similarly, gallic acid derivatives (GADs) have also been found in a number of phytomedicines with diverse biological and pharmacological activities, such as ROS scavenging, interfering the cell signaling pathways, and apoptosis of cancer cells

Flavones

Compound (**5**) was identified as dihydroquercetin $[M-H]^-$ m/z at 303.0510 based on the fragment peaks at m/z 285 [M-H-H_2O], m/z 275 [M-H-CO] and m/z 151 [M-H-RDA cleavage] (Chen *et al.,* 2016).

Other Compounds

Other compounds identified to be present in the plant extracts include, Malic acid (**4**). Malic acid, a component of compound (5) with a precursor at [M-H]- m/z 134, is discovered to be present in both the leaves and fruit extract employed in this study. Molecular loss caused the fragment peak at m/z 115 [M-H-H_2O] to appear. According to earlier research, malic acid has a sour, acidic taste. This aids in removing dead skin cells when applied to the skin. Its sourness also stimulates salivation in people who have dry mouths. Malic acid also takes part in the Krebs cycle. To create energy, the body goes through this procedure. A dry mouth is frequently treated with malic acid. It is also used to treat several other conditions, including fibromyalgia, fatigue, wrinkles, and acne (WebMD, 2022).

Biological activity

Saliu and Olabiyi (2017) reported on the antioxidative potential of phenolic compounds in protecting the human body system from free radicals. The extract's phenolics had the ability to remove free radicals, chelate metallic catalysts, activate antioxidant enzymes, reduce alpha tocopherol radicals, and block oxidases (Amic, Davidovic, Beslo, & Trinajstic, 2003). They may also improve food quality by changing taste, fragrance, color, and flavor (Memnune et al., 2009). The presence of flavonoids and phenolics (gallic acid, chlorogenic, caffeic acid, rutin, quercetin and kaempferol) in the extract may also contribute to lowering cellular oxidative stress and inhibit α-amylase, α-glucosidase, acetylcholinesterase and butylrycholinesterase activities (Adefegha & Oboh, 2015). Singh et al. (2012) reported that hydroxycinnamic acids and their derivatives have significant impacts on blood sugar levels. It may provide therapeutic benefits for people with diabetes. This finding corroborates the result from this study where hydroxycinnamic acids were found to be present in both polyphenolic extracts of the two plants employed giving rise to the good antioxidant activity in the alpha-amylase assay.

Singh et al. (2012) reported that hydroxycinnamic acids and their derivatives have significant impacts on blood sugar levels. It may provide therapeutic benefits for people with diabetes. This finding corroborates the result from this study where hydroxycinnamic acids were found to be present in both polyphenolic extracts of the two plants employed giving rise to the good antioxidant activity in the alpha-amylase and alpha-glucosidase assays. Another biologically useful class of compounds in plant extracts is hydroxybenzoic acids. The inhibitory action of 2-Hydroxybenzoic acid (4-Chloro benzylidine)-Hydrazide which is a derivative of hydroxybenzoic acid was found to remarkably reduce the activity of enzyme as acarbose. Comparable results were reported, greater orders of -amylase inhibitory effects were seen. The aforementioned findings, however, indicate that the synthetically produced 2-hydroxy benzoic acid benzylidene hydrazide-based Schiff base derivative may be more effective antidiabetic particles at inhibiting carbohydrate-digesting enzymes and may be a useful strategy in the treatment of diabetes (Anusuya *et al.,* 2020). This assertion explains the increasing trend in the activity of *Solanecio Biafrae* Fruit extract in this study.

CONCLUSION

The antioxidant, antidiabetic and LC-MS identification of compounds profiling in the leaves extract of *Crassocephalum crepioides* was carried out in this study. Six (6) compounds of different chemical classes and of diverse biological application were identified in the plant extract base on their m/z ratio. The compounds classes are hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and other benzoic acids. From the biological activity of the extracts, *C. crepioides* exhibited the good antidiabetic activity which increased with corresponding increase in the dosage of the extract. The best activity was achieved at 250 μg/ml (16.9934641) in the ABTS assay which was far better than that of the control drug acarbose at the same dosage. Similarly, in the antioxidant assay carried out in this study, *C. crepioides* also exhibited the best activity across all assay when compared with the control drug ascorbic acid.

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