Rapid Identification of *Cunninghamella bertholletiae's* Toxins/Secondary Metabolites via a Fermentation Technique

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***Abstract*— Toxins/secondary metabolites produced by a cyanide tolerant/resistant fungal strain, i.e. *Cunninghamella bertholletiae*, were produced through a fermentation technique. Fungal mycelia (0.05 – 0.4g) was inoculated and fermented in a 25 mL nutrient broth medium for 168 hrs at 37 °C in 40 mL Erlenmeyer flasks in a shaking incubator set at 70 rpm. A volume (5 mL) of the fermented extracts were filtered through a membrane, centrifuged, mixed with chloroform and dried-up through nitrogen blow-down prior reconstitution using 100% analytical grade methanol. Samples were analyzed for secondary metabolites identification using Liquid Chromatography-Mass Spectroscopy (LC/MS-TOF 6230). The results revealed the production of secondary metabolites; Silibinin and 11-Ketotestosterone from a cyanide resistant and/or tolerant *C. bertholletiae*.**

***Keywords* —** Cassava, *Cunninghamella berthollethiae*, Liquid Chromatography-Mass Spectroscopy, Secondary metabolites.

I. Introduction

A shelve life of cassava during a postharvest period is often threatened by spoilage from fungal and bacterial attacks [1]-[3]. Fungal species such as *Cunninghamella* sp., *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp. produce and release secondary metabolites which negatively affect the quality of the produce [2], [4], [5].

Most of the produced secondary metabolites, negatively affect agricultural products, thus leading to losses [6]-[8]. Toxins are biosynthetic compounds (metabolites) produced by toxicogenic fungal species and other microorganisms under natural or a controlled environment during their exposure to harsh environmental conditions [9]. These compounds, also known as secondary metabolites, are also used in the industry, i.e. in the production of some hormonal compounds [10]-[12]. Toxins are mostly found in molds produced by several fungal species but, the most known fungal producers of toxins are; *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp. [13]. There are a variety of secondary metabolites produced by fungal species, some of these toxins are hazardous to plants, humans and animals, e.g. Aflatoxins, Fumonisins, DON, Ochratoxins, etc. [1], [13], [14]. Some types as elucidated in [15]-[17], i.e. Ochratoxins (A, B and C), Aflatoxins (AFB 1, AFB 2, AFG 1 and AFG 2), Fumonisin (FB1) [9], [16], [18], [19], and were determined to be detrimental to human health. Although, secondary metabolites occurrence in the environment and in agricultural food products as well as feeds, are a serious concern, not only to human health clinical outcomes [20]-[22], there is potential for huge economic losses due to medical health claims and production losses from agricultural product producers [6], [23], [24]. Some secondary metabolites observed in agricultural produce include 11Ketotestosterone (3) and Silibinin which have some clinical and health advantages [25]-[28]. Previous studies have revealed that *C. bertolletiae* could be cyanide resistant and be able to biodegrade the compound (cyanide) [29] but, there is little or no evidence in literature available on secondary metabolites produced by cyanide resistant *Cunninghamela bertholletiae* sp.

II. Material and Methods

A. Fungal Isolation, Identification and Characterization

Fungal (*Cunninghamella bertholletiae*) isolation, identification and characterization was done using a culture-based technique, from cassava-cultivated soil that was sprinkled into a Potato Dextrose Agar (PDA) (Merck, Germany) and incubated for a duration of 168 hrs, at room temperature. After several sub-culturing cycles, a pure colony of *C. bertholletiae* was aseptically transferred into Nutrient Broth medium (Merck, Germany), in a 40 mL airtight multiport shake flasks. The cyanide concentration (as KCN) was set at 100 mg CN^-/L and the culture was incubated at 37°C for 168 hrs, followed by spread-plating on PDA and the PDA plates were incubated at room temperature for a further 168 hrs. This was done to assess fungal/ organisms resistance and/or tolerance to

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cyanide. A pure fungal strain was isolated from the PDA and re-inoculated in Nutrient Broth for 24hrs for subsequent DNA extraction and sequencing procedures, which were conducted as described elsewhere [29]. The generated nucleotide sequence was analysed using CLC main workbench 7 followed by a BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Centre for Biotechnology Information) and was identified and assigned the accession number: KT275316.

B. Secondary Metabolites Extraction and Identification

C. bertholletiae was grown on a PDA for a period of 168 hrs and thereafter, the mycelia was removed from the PDA using a surgical blade. A mass (0.05 – 0.4 g) of the extracted mycelia was inoculated into a 40 mL Erlenmeyer flask containing 25 mL of Nutrient Broth (Merck, Germany), followed by incubation in an orbital shaker at 37 °C for a period of 168 hrs. Extracts (suspension) were filtered using a No 5 Whatman filter paper in order to obtain extracts free of spores and mycelia [30]. The liquid fraction was transferred into centrifuge tubes followed by centrifugation at 5000 rpm for 10 min using a Megafuge 1.0 (Kendrol Laboratory products, Germany). The supernatant was transferred into clean 20 mL sampling tubes while the pellets were discarded.

C. Mycotoxin (Secondary Metabolites) Extraction and Detection

Toxins (secondary metabolites) extraction was done using a modified liquid-liquid extraction method [31], [32]. 5 mL of the extracts were transferred into clean tubes containing 5 mL of 100% chloroform (Sigma Aldrich, Germany) and the contents were mixed by inversion 3-5 times, followed by centrifugation at 5000 rpm for 10 min using a Megafuge 1.0 (Kendrol Laboratory products, Germany). The aqueous phase containing chloroform was transferred into clean tubes while the Nutrient Broth phase was discarded [33]. The aqueous phase was evaporated in a sterile environment at room temperature for 24 hrs followed by complete blow-down using nitrogen (N_2) gas (Afrox, South Africa). Sample contents were reconstituted (by dilution) using 1 mL of 100% methanol (Sigma Aldrich). 1 mL of the reconstituents was transferred into a LC/MS analysis vial using a 1 mL glass syringe and 0.2 µm Whatman syringe filter. The samples were analysed using LC/MS-TOF 6230 with the mobile phase flow rates as described in Table 1.

TABLE I: LC/MS ELUENTS AND ANALYSIS PARAMETERS

Gradient	A (H_2O)	B (MeOH)	Flow (µL/min)
0	85	15	0.4
30	0	100	0.4
33	0	100	0.4
45	85	15	0.4
50	85	15	0.4

A ToF LC/MS used for analysis/identification of mycotoxins had the following operational parameters; MS Interface Ionisation: Electronspray ionisation (ESI), High vacuum pressure of $1.16x10^{-7}$ Pa (Torr), Dry gas temperature of 350 ^oC, Nebulising pressure of 15 psig, Dry gas flow of 8.0 L/min, A positive, acquisition mode, a ESI capillary voltage of 3500 kv, a mass range scan between 112.9855-966.0007, ToF flow rate of 3 mL/min, samples injection volume of 5µL, with a C8 column symmetry (150 x 3.9 mm, 5µm at the temperature of 30^oC); a Symmetry C8 guard column: 3.9 x 20 mm, 5µm while, a mobile phase constitute of solvent A (Water), solvent B (Methanol) which were of analytical grade. The identification of the toxins/secondary metabolites from *C. bertholletiae* were done according to *m/z* ratio of library toxins and secondary metabolites.

III. RESULTS

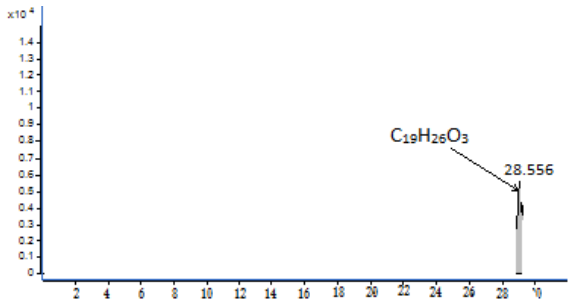
C. bertholletiae's toxins/secondary metabolites production was assessed via a fermentation technique in nutrient broth medium with the separation process being done using chloroform, which was followed by the total evaporation of the extracts and subsequent reconstitution in absolute methanol. The compounds listed on Table II were identified based on their molecular features and the extracted ion chromatograms (EICs) are presented in Figure 1(A, B).

Additionally, the compound identification is important due to their impacts on humans and animals. Thus, 11-Ketotestosterone in human's health is used a main source of androgen; known as a human's growth and reproduction hormone; as well as in breast cancer treatment [25], [28], [34] while, Silibinin is an antioxidant, antiviral agent which is used for cancer treatment and treatment liver diseases [26], [27], [35].

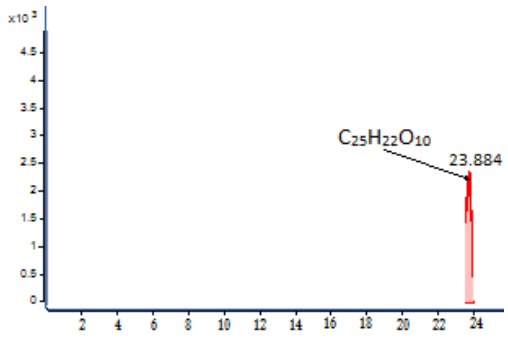
TABLE II: C. BERTHOLLETIAE'S SECONDARY METABOLITES IDENTIFIED USING LC/MS

Compounds	Formula	Molar mass (g/mol)	(m/z) to charge ratio
11-Ketotestosterone (3)	$C_{19}H_{26}O_3$	302.42	303.19
Silibinin	$C_{25}H_{22}O_{10}$	482.44	483.12

The detection of the secondary metabolites on LC/MS was adopted from a method developed by [14], [16], [35]-[37], from which the compound produced moderate signal under a positive acquisition mode. Its chromatographic separation during Electron Spraying Ionization (ESI) of both phases (forward and reverse) revealed a clear peak, while, the compound counts (level) were detected (Figure 1A, B).



A



B

Fig. 1. Molecular features and the extracted ion chromatograms (EICs) for secondary metabolites: (A) 11-Ketotestosterone (3), (B) Silibinin

A. *11-Ketotestosterone (3)*

11-Ketotestosterone (3) was detected in a fermentation medium for the cyanide resistant organism. An LC/MS analysis of the sample on a positive mode of acquisition as well as a chromatographic separation through Electron Spraying Ionization (ESI) resulted in a clear peak although its concentration in the sample was low. Thus, the compound counts was 0.5 x10^4 ppm while, its acquisition time was 28.520 min (Figure 1A).

B. *Silibinin*

Similarly, the Silibinin was also extracted from the medium used for fermentation; however, the compound detection through a positive acquisition mode in both the ESI phases resulted in a clear chromatograph peak with a resultant count of approximately 2.5x10^3 ppm while, the time of acquisition was 23.884 min (Figure 1B). The observed detection count of Silibinin was higher than that of 11-Ketotestosterone (3).

IV. Discussion

It is important to observe that secondary metabolites such as; 11-Ketotestosterone are one of human's main source of androgen which is a hormone which facilitates men and females growth and reproduction [25]. Previous studies revealed that androgen receptor activation plays a major role in a treatment of breast cancer [25], [28], [34].

Silibinin is an antioxidant, antiviral, and cell catalyst (pSTAT3) used in the treatment of lung, prostate, breast, gastric and skin cancer, liver disease including the treatment of other malfunctioning humans organs [26], [27], [35], [38].

This study modified the method of [39] for toxins extraction using a fermentation method to obtain fungal *C. bertholletiae* extracts for LC/MS analysis of toxins/secondary metabolites because, the latter appeared easier, faster and cheaper. Thus, the analyses revealed a successful identification of secondary metabolites 11-Ketotestosterone 3 and Silibinin, respectively.

Therefore, it was clear that the acquisition time of the compound (11-Ketotestosterone 3) was high (28.520 min) due to its low concentration in the sample and molecular structure. On the other hand, a high concentration of Silibinin in the sample enabled its rapid detection at a short period of time (23.884 min).

The detected secondary metabolites play a significant role to living organisms, and especially in humans and animals. These compounds may be beneficial and also hazardous to humans in higher concentrations.

V. Conclusion

This study revealed the production of secondary metabolites from a cyanide resistant and/or tolerant *Cunninghamella bertholletiae*. Silibinin and 11-Ketotestosterone were successfully produced and identified.

It is, however, recommended that future research should be directed at the evaluation of the secondary metabolite on different *C. bertholletiae* strains and other related fungal organisms.

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