

*Full Length Research Paper*

# Partial chemical characterization of the yeast extracts *Lachancea thermotolerans* CCMA 0763

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The fungi are living organisms of great importance around agriculture, food industry, and the pharmaceutical industry. The usage of these microorganisms in agriculture is for the biocontrol of diseases in plants of commercial interest. Some yeasts can fight phytopathogens through secondary metabolites produced, inhibiting their development. Some species show positive results in the control of pathologies in different cultures. Yeasts have been used as biocontrol agents, and among them, a *Lachancea (Kluyveromyces) thermotolerans*, which is present in plants (such as grapes), soil and insects, can be adapted to different environments and, possibly perform biological control, although it is not known its mode of action. This work aimed to analyze and identify secondary metabolites used by the yeast *L. thermotolerans* CCMA 0763 isolated from commercial grapes in the Agricultural Microbiology Laboratory (Federal University of Lavras-MG/BR), using the analytical method of Ultra High-Efficiency Liquid Chromatography (UHPLC) (HRMS) and Tandem Mass Spectrometry (MS/MS). Four heterocyclic compounds of the Alkaloids class were identified, three (03) natural: 4-Hydroxyquinoline, Xanthine and Calistegine A3, and one (01) synthetic: Clausehainanine C. Therefore, these compounds can be tested against phytopathogenic microorganisms.

**Key words:** Yeast, biological control, secondary metabolite, ultra high-efficiency liquid chromatography (UHPLC), mass spectrometry.

## INTRODUCTION

Non-*Saccharomyces* yeasts are sources of complex aromatics, glycerol content and responsible in ethanol reduction and polysaccharide concentrations in industrial processes. Its main functionality is associated with the production of alcoholic beverages from fermentation

processes (Contreras et al., 2014; Belda et al., 2015, 2017; Domizio et al., 2017). This fungus is mainly in the production of wine, on substrates containing hexoses, playing an important role in the characteristics of the drink not producing spores or allergenic toxins; and are

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not nutritionally demanding. They present rapid growth, they do not present risks to the consumer and they act as antagonists, either through competition for nutrients, antibiosis or hyperparasitism (Domizio et al., 2017; Mehloimakulu et al., 2014, 2015). However, its use is not limited to wines, beers, breads and cheeses productions, but on other products for example control of phytopathogens. The good performance of yeasts as biocontrol agents is due to the different modes of action of these microorganisms against phytopathogens. Among these, we can highlight the competition with phytopathogens for space and nutrients, mycoparasitism, antibiosis, resistance induction in plants and the production of antimicrobial toxins and alcohol by yeasts. Understanding these biocontrol mechanisms is essential to improve the action of these microorganisms in combating plant diseases (Hatoum et al., 2012; Muccilli and Restuccia, 2015). Biological control is an important management strategy for fungal diseases, with the basic principle of using antagonistic microorganisms to reduce and/or keep the population of a phytopathogen below levels that cause economic loss (Carmona-Hernandez et al., 2019). Organic control using biological agents against mycotoxins is considered a safer option until now, gaining popularity in the food industry (Tsitsigiannis et al., 2012). In fact, the application of yeasts (cells and their volatiles) and yeast derivatives has great potential to minimize the economic losses caused by mycotoxigenic fungi. Several of yeast and bacteria species have been identified as having effective fungi biological control activities at pre- and post-harvest, where they were tested against toxigenic fungi to inhibit their growth and mycotoxin synthesis (Catara, 2007; Haiissam, 2011). Within the microorganisms tested, the non-saccharomyces yeasts are promising antimicrobial and bio-remediation agents (Übeda et al., 2014). However, the antimicrobial properties associated with yeast are linked to the production of killer toxins. Killer toxins have been identified as glycosylated proteins that bind to specific receptors. Their strains were called killer yeast. Killer yeasts secrete lethal toxins to and their strains are insensitive to their own toxins (Alturki et al., 2019; Mannazzu et al., 2019).

In addition, the biological properties associated with this type of microorganism are not limited only to the production of proteins, but in the direct and/or indirect interactions between the different strains through the physical-transformed changes in the environment caused by the reaction of one strain to another. The effect of a specific yeast strain on its environments is unique suited to its metabolites and their respective (Roullier-Gall et al., 2020). Thus, it is evident that non-saccharomyces yeasts have a favorable metabolic engineering for the production of new and well-known compounds with wide biological activity (Kulkarni et al., 2015; Liu et al., 2020). Currently, thousands of secondary metabolites have been identified from various living organisms, mostly plants. However, it

is estimated that this number is higher, being, in part, limited to analytical techniques. With the instrumental advance, compounds with a high degree of difficulty in analysis, whether due to their abundance, structural diversity or the complexity of the matrix, have been constantly identified (Zhao et al., 2013). Among the technological tools used to identify these molecules, is Ultra High Efficiency Liquid Chromatography (UHPLC) (HRMS), to analyze the yeast metabolome, for its excellent resolution, mass accuracy, new possibilities for improving analytical methods for matrices complex samples (ingredients, food and biological samples) and a wide range of spectrometric data (Motilva et al., 2013). Thus, the present work aimed to partially characterize the secondary metabolites produced by the yeast *Lachancea thermotolerans* CCMA 0763, isolated from commercial raisin, using the analytical technique of (UHPLC)(HRMS) and Tandem Mass Spectrometry (MS/MS).

## MATERIALS AND METHODS

### Crude extract obtainment

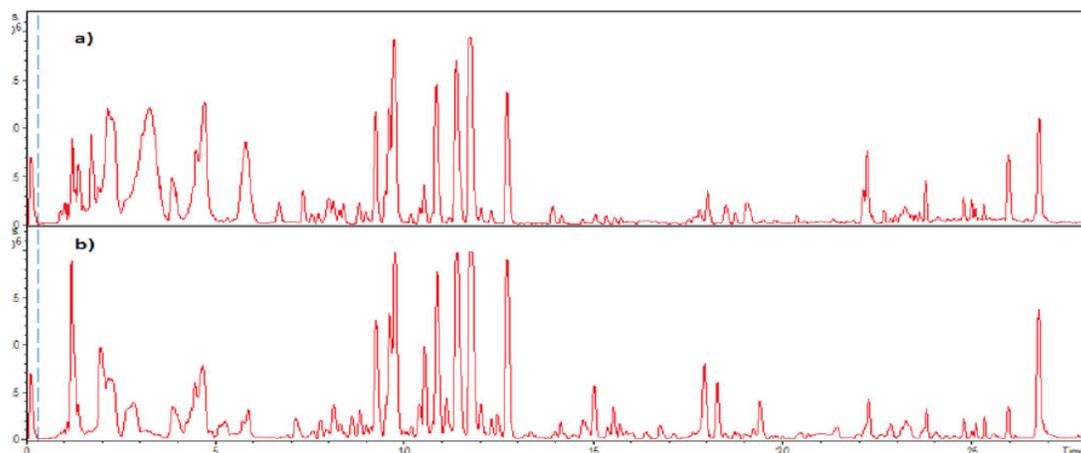
The yeast *L. thermotolerans* CCMA 0763 (Sample) belonging to the Agricultural Microbiology Culture Collection at the Federal University of Lavras - Minas Gerais/Brazil was grown in a Petri dish containing Potato-Dextrose-Agar (PDA) (Culture Medium) for 2 days and inoculated in 500 ml Erlenmeyers containing Potato-Dextrose (PD) with pH 6.8 and incubated without shaking for 21 days at 28°C (Quality Control). The mycelium was separated from the fermented broth by filtration in cotton and hydrophobic gauze and the aqueous phase was centrifuged at 2750 rpm for 15 min. The liquid-liquid extraction was performed with Ethyl Acetate in the proportion of 1:3 (Ethyl acetate: fermented broth) in a separating funnel. This step was repeated three times. The solvent was collected and evaporated in a Rotary Evaporator (Tecnal TE-210) at 400°C and the Crude Extract (1.014 g) subjected to Ultra High Efficiency Liquid Chromatography Analysis (UHPLC) (HRMS) and Tandem Mass Spectrometry (MS/MS). The same procedure was used for the Quality Control sample.

### Chemical analyses

The solvents used were: Acetonitrile grade UHPLC-MS (JT Baker-Phillipsburg, NJ, USA), Deionized Water from a Millipore Milli-Q water reagent system (Millipore, Bedford, MA, USA) and Analytical grade Formic Acid (85%). The extract of the yeast *L. thermotolerans* (1.0141 g) was solubilized in methanol and the concentration of the solution corrected to 1.0 mg. mL<sup>-1</sup>. For the Mass Spectrometry Analysis, a volume of 1.0 ml of Yeast Extract was collected and 10.0 µl of 0.1% Formic Acid (v/v) (Analysis in positive mode) was added and stored in a vial for analysis.

### Liquid chromatography

The extracts (Sample and Control) were analyzed by Ultra-High Performance Liquid Chromatography (Shimadzu, Nexera X2, Japan) coupled to a High Resolution Mass Spectrometer (Impact II, Bruker Daltonics Corporation, Germany) equipped with an ionization source by electrospray. The chromatographic separation was performed with an Acquity UPLC® CSHTM C18 column,



**Figure 1.** UPLC-Q-TOF/MS BPI chromatogram in mode positive from Quality Control (a), and yeast *Lachancea thermotolerans* (b).

particle size of 2.1  $\mu\text{m}$ , with 2.1  $\times$  100 mm (Waters, Ireland) and flow rate of 0.200  $\text{ml min}^{-1}$ . The gradient of the solvent mixture A ( $\text{H}_2\text{O}$  with 0.1% (v/v) formic acid) and B (acetonitrile with 0.1% (v/v) formic acid) as follows: 5% B 0-1 min, 50% B 1- 5 min, 95% B 5-10 min and maintained at 95% B 10-16 min, 5% B 16-18 min and maintained at 5% B 18-32 min at 40°C.

### Mass spectrometry

The ionization source was operated in positive ionization modes and set to 4500V with a potential displacement of the final plate of 500V. The drying gas parameters were adjusted to 8  $\text{L min}^{-1}$  at 180°C and the nebulizer gas pressure was set to 4 bar. The data were collected from  $m/z$  50 to 1300 with an acquisition rate of 5 Hz and the 4 most intense ions were selected for automatic fragmentation (Auto MS/MS).

### Data processing and statistical analyses

The software used for data processing for peak detection, multivariate analysis and identification was Data Analysis 4.0 (Bruker, Germany). The specialized databases were: MoNa (<http://mona.fiehnlab.ucdavis.edu/>), ChemSpider (<http://www.chemspider.com/>) and Metlin (<https://metlin.scripps.edu/>) for the identification of compounds. The search parameters in the online databases were: precursor mass with error  $\leq$  10 ppm and fragment tolerance  $\leq$  10 ppm. Principal component analysis (PCA) and projection discrimination in an orthogonal latent structure (OPLC-DA) were generated using the ProfileAnalysis 2.1 software.

## RESULTS AND DISCUSSION

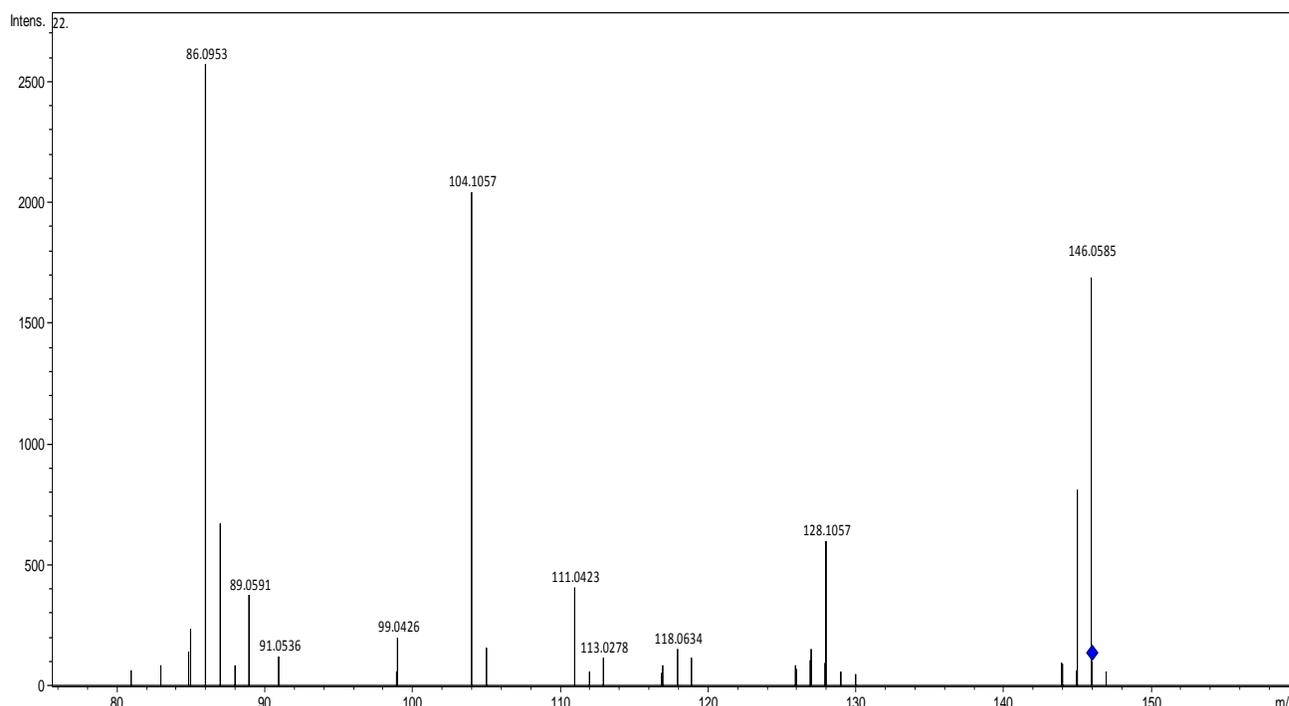
Figure 1 represents the diversity of the chemical profiles of *L. thermotolerans* and Quality Control extracts in the positive mode chromatogram generated from the UHPLC-HRMS. The chromatographic run had an injection time of 28.0 min with 4.0 min for column balance and cleanliness.

It was revealed in this first analysis that in the time between 1.0 to 6.0 min, the two chromatograms behave differently in relation to the intensity and areas of the peak. This is also seen in the time between 14.0 and 25.5 min. However, the chromatographic profile is very similar between the Control and the Sample, little difference between them. However, UHPLC-HRMS analyzes provide coverage of the chemical space based on MS and MS/MS data and are capable of presenting the best differentiation between Sample and Control. For this experiment, the molecular masses studied were 50 to 1300 Dalton (Da). This mass distribution of precursors was based on studies in the literature (Maciá-Vicente et al., 2018).

The global chemical space observed in the complete data set comprised eight thousand exclusive MS/MS spectra after the first performed for a sample and control. To minimize and discard potential noise or artifacts present in the UHPLC-HRMS analyzes, three boundary conditions were imposed: first, the removal of the spectra present only in the control or in the whites; second, consider the final spectra that occurred only in the sample; third, consider spectra with acquired MS/MS, an important identification of the compounds in the sample. All spectral data were collected and processed using centralization, isotopic pattern, filtering, retention time and peak recognition methods to generate a datamatrix including sample identity, ion identity and ion abundance. The result of this mathematical deconvolution procedure provided 126 exclusive ions from the sample. The software used for data processing for peak detection, multivariate analysis and identification was DataAnalysis 4.0 (Bruker, Germany) and Software ProfileAnalysis 2.1. For the identification of compounds, specialized Database sites were used, such as: MoNa, ChemSpider and Metlin. Other parameters, such as fragmentation

**Table 1.** Results of high resolution UHPLC-HRMS mass spectrum analysis.

Library	Compound	[M+H] <sup>+</sup> m/z	RT (min)	Exact mass	Formula	MS/MS
Mona	1	1.460.585	6.49	1.450.528	C <sub>9</sub> H <sub>7</sub> NO	128.1057; 118.0634; 111.0423; 104.1057;99.0426; 86.0953
Mona	2	1.530.395	2.70	1.520.334	C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O <sub>2</sub>	136.0133; 110.0343 142.0853; 130.0854; 124.0748; 112.07
Goldmann et al. (1990)	3	1.600.958	1.03	1.590.895	C <sub>7</sub> H <sub>13</sub> NO <sub>3</sub>	52; 103.0385; 94.0648; 86.0600; 82.0648
Ma et al. (2018)	4	2.921.332	0,34	2.911.259	C <sub>19</sub> H <sub>17</sub> NO <sub>2</sub>	246.1301; 200.1253; 158.1159; 1121110

**Figure 2.** ESI-MS/MS spectrum for 4-Hydroxyquinoline.

score and bibliographic record on the occurrence of the molecules, were also considered for disambiguation. The main exclusion factors used for identification were: mass error less than or greater than 10 ppm to decrease the number of candidates, fragmentation profile to justify and prove the identification and the probability of ionization by ESI.

This attention with several exclusion factors is associated with the possibility of compounds within a mass range of 0.5 to 5 ppm, which generally have very different numbers of carbon atoms and the biosynthesis of units with a common elementary composition that provides information limited. Thus, mass accuracy and isotopic ratio, are almost orthogonal parameters that can be used to exclude hypothetical elementary compositions,

but do not establish a consistent identification (Nielsen et al., 2011). In these cases, MS/MS, or preferably by Sequential Mass Spectrometry (MS<sub>n</sub>), with subsequent fragmentation patterns is more efficient, assuming that reference standards are available to model the fragmentation of the class of compounds (Sharma et al., 2007). Thus, all precursors were submitted to the parameters presented here to support their identification. Four compounds were identified (Table 1), with their ESI-MS/MS spectra, respectively (Figures 2 to 5).

All compounds identified are alkaloids (Figure 6). They have nitrogen atoms and a differentiated fragmentation profile in their chemical skeleton. The compound identified as 4-Hydroxyquinoline belongs to the group of nitrogenous heterocyclic compounds of the quinoline

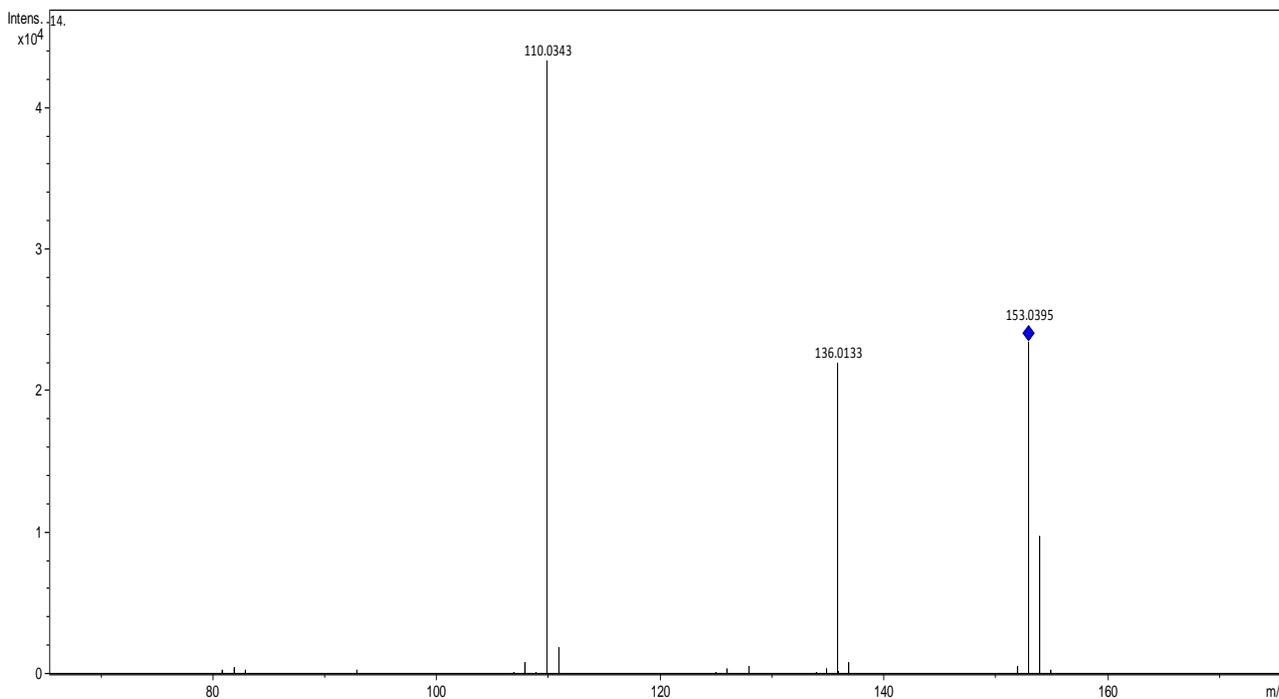


Figure 3. ESI-MS/MS spectrum for Xanthine.

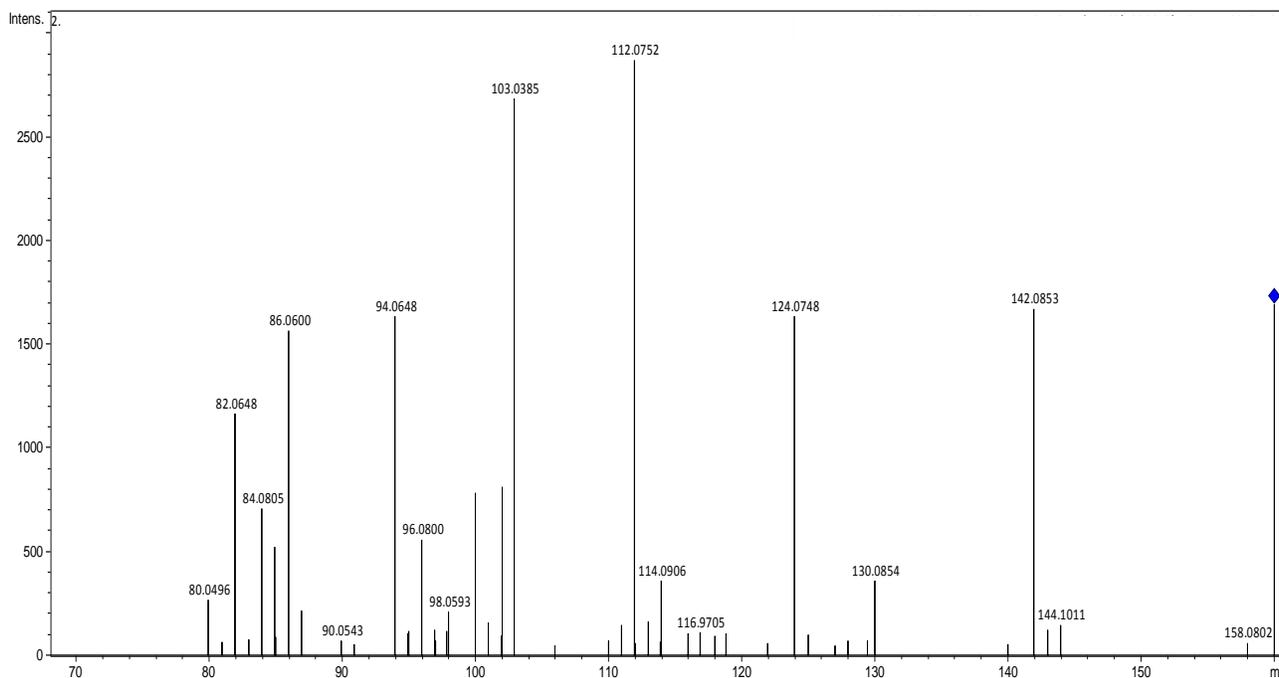
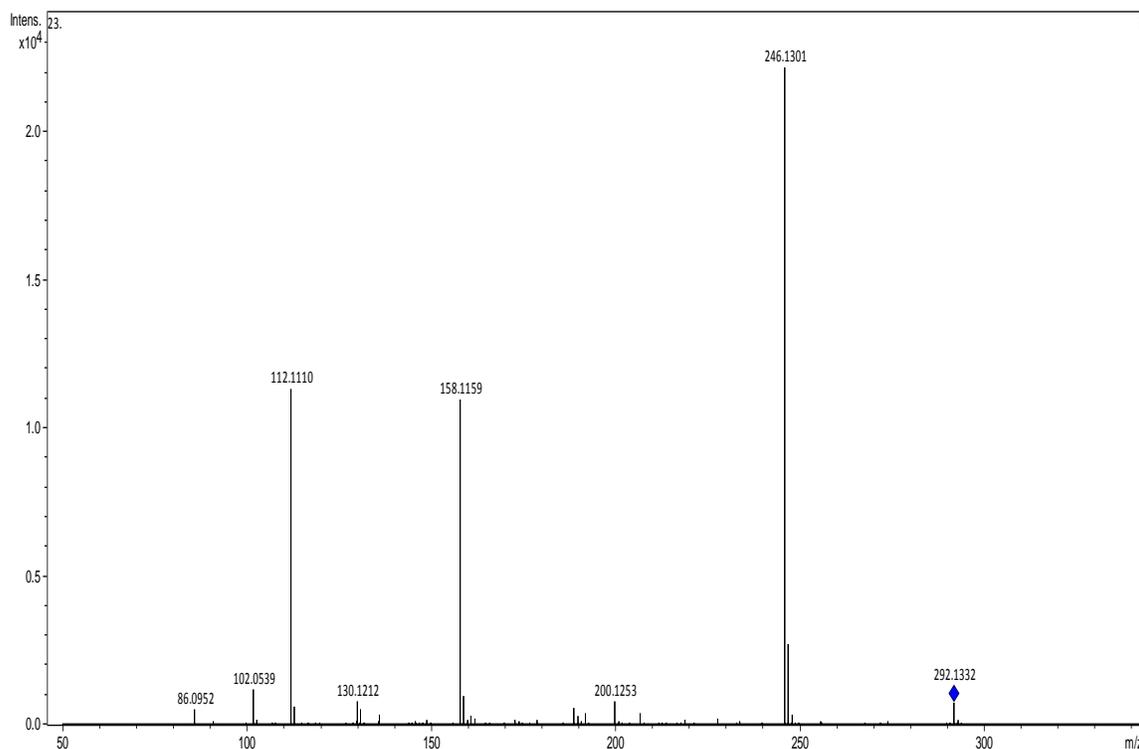


Figure 4. ESI-MS/MS spectrum for Calystegine A3.

alkaloids class. They play an important role in several areas of knowledge and have a wide range of natural substances. Quinolones are molecules structurally

derived from the heterocyclic Quinoline, being distributed in nature as a product of the secondary metabolism of several species of plants and fungi, mainly in species of



**Figure 5.** ESI-MS/MS spectrum for Clausehainanine C.

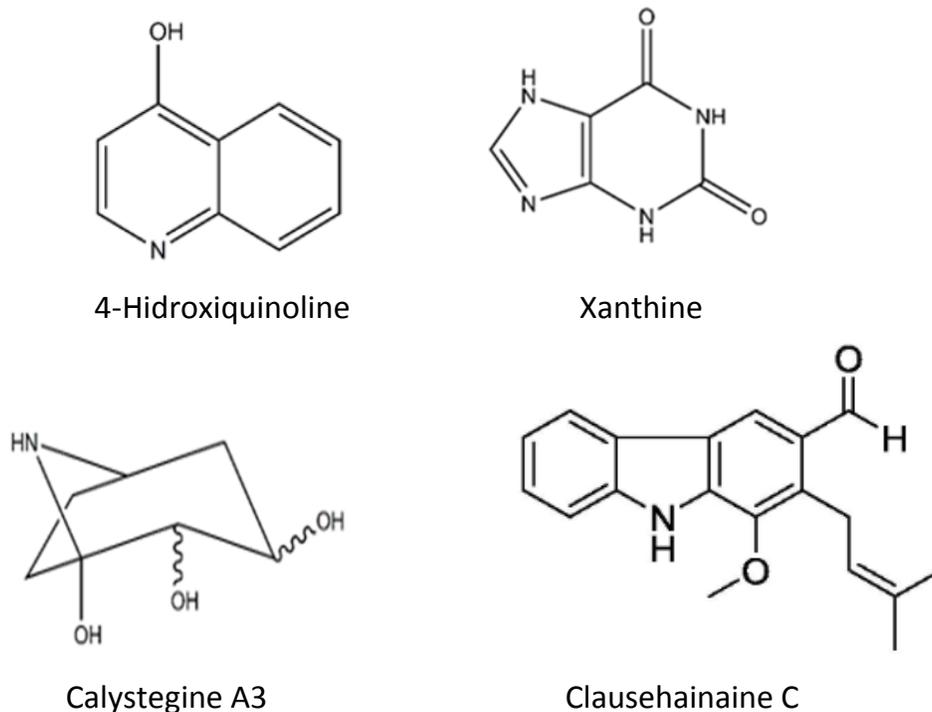
the family *Rutaceae* (Suárez et al., 2011). Several derivatives of Quinolone have been isolated from this family with analgesic, amebicidal, antiviral, herbicidal and fungicidal activities (Barrera and Suarez, 2007). The compound 8-Hydroxyquinoline and its derivatives are a subclass of Quinolines with a wide variety of biological activities. They have been used as a fungicide in agriculture and a preservative in the textile, wood and stationery industries (Oliveri and Vecchio, 2016).

Xanthines are a class of Purine Alkaloids found in many different plants, including yerba mate (1,3- and 3,7-Dimethylxanthine), coffee (1,3,7-Trimethylxanthine) and cocoa (Athayde et al., 2000). Another particularity associated with these compounds is their biological function in conjunction with the enzyme Xanthine A dehydrogenase (XDH), a Hydroxylase containing Molybdenum, being important for regulating the metabolism of Purines and Uric Acid responsible for the regulation of *Arabidopsis* aging and resistance to stress, peas, corn and grapes (Barabás et al., 2000; Zdunek-Zastocka and Lips, 2003; Brychkova et al., 2008; Werner and Witte, 2011; Shuanghong et al., 2017; Han et al., 2020).

Calistegines are a group of alkaloids (glycoalkaloids) initially discovered in the roots of cultures of *Calystegia sepium* and *Atropa belladonna*. The chemical structures contain a Nortropene ring system with 3-, 4-, or 5-Hydroxyl groups (Calistegines A, B or C, respectively), located in various positions with different stereochemistry

(Aminocetal functionality), and an Amino bridge group (Asano et al., 2000). These compounds were identified in several genera such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Escopolia* belonging to the *Solanaceae*, *Convolvulaceae*, *Erythroxylaceae* and *Brassicaceae* families (Schimming et al., 2005; Brock et al., 2005, 2006; Torres-Romera et al., 2019). The biological activities associated with polyhydroxylated alkaloids are their levels of toxicity to bacteria, fungi, viruses, insects, animals and humans (Friedman, 2006).

Carbazolic Alkaloids are characterized by a basic aromatic tricyclic skeleton consisting of a central Pyrrole ring fused with two Benzene rings. Carbazol itself was originally isolated from the Anthracene fraction of coal tar (Greger, 2017). They have been found in bacteria, myxomycetes, fungi, sponges, tunicates and in the related plant families *Apocynaceae* and *Loganiaceae*. The vast majority of Carbazoles, comprising more than 330 derivatives, have been shown to be derived from 3-Methylcarbazole as a common precursor (Ryvolova et al., 2012). The vast majority of Carbazoles, comprising more than 330 derivatives, have been shown to be derived from 3-Methylcarbazole as a common precursor. This type of Carbazoles was called Fitocarbazoles (Chakraborty and Roy 2003). However, there is no scientific evidence from recent studies related to the application of this compound in the area of agriculture, so it can be the object of research to be investigated.



**Figure 6.** Compounds identified in the extract of *Lachancea thermotolerans*.

## Conclusion

The crude extract of secondary metabolites from the yeast *L. thermotolerans* presents eight thousand exclusive MS/MS spectra through the UHPLC-HRMS analysis. These data were included in the mathematical deconvolution procedure, obtaining 126 exclusive ions from the sample. The analytical method of UHPLC-HRMS has been a high performance tool for the identification of compounds and biomolecules of great structural diversity for the analysis of secondary metabolites. Thus, four components of the class of heterocyclic alkaloids were identified, three (03) naturals: 4-Hydroxyquinoline, Xanthina and Calistegina A3; and one (01) synthetic: Clausehainanina C from the crude extract of *L. thermotolerans*.

These compounds present, through the literature: antimicrobial, antiproliferative, inhibitory and regulatory activities, applied in the most diverse areas of knowledge, being able to be tested as biocontrol agents in antagonistic tests of biological tests against phytopathogens that cause fungal and bacterial diseases in agricultural cultures

## ACKNOWLEDGMENT

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## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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