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Screening for Metabolites in Earthworms Exposed to Polluted Landfill Dumpsites Using LCMS in Gombe Metropolis

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ABSTRACT

Metabolomics is now the state of an art used to study environmental effects such as environmental stress, pollution and climate change on the health of living organisms that lives in an environment. The study aimed to screen metabolites in earthworms exposed to polluted landfills dumpsites via identification of metabolites present in the earthworm (Bio indicators) and identify to compare metabolites found in different earthworm groups using LCMS metabolomics. Metabolites were extracted from earthworms as whole organism extracts; earthworms were washed with distilled water, flash-frozen in liquid nitrogen, and homogenized by bead-beating using mortar and pestle in 1 mL of (50:50) cold methanol/water. The content were transferred into eppendorf tube (2ml) and vortexed for (1min), centrifuged at 1800 rpm for 20 min, and the supernatant were transferred to a new tube for cleanup with 500 µL of ice-cold chloroform. The aqueous layer were dried and stored at −4°C. All samples collected were subjected to gas chromatography/ mass spectrometry (LC/MS). The RAW LC-MS data were three replicate from two dumpsites; Liji landfill dumpsite labelled L1, L2, and L3, Inex cleaners limited dump sites opposite FCE (T) Gombe labelled F1, F2, and F3. The platform, MZ mine software version 2.53 was used to processed data for LC MS based metabolomics. The outcome of this research shows the potential marker compounds that brought about the separation of the groups Liji dumpsite (L) and Inex cleaners dumpsite (F) by PC1 and PC2. It was observed that most of the identified compounds belong to (F) dumpsite, with few belonging to (L) dumpsite. The VIP metabolites which were considered as most-ranked metabolites were: 4Z,7Z,9E,11E,13Z,16Z,19Z-docosaheptaenoic acid, 1-Phenyl-1,3-dodecanedione, 5-(10'-Phenyldecyl)-Resorcinol, Beta-estradiol and 1-(7Z,10Z,13Z-hexadecatrienoyl)-3-O-beta-D-galactosyl-sn-glycerol.

Keywords: Biochemical, liquid chromatography, metabolomics, dumpsites, multivariate analysis.

INTRODUCTION

Earthworms are at the forefront of soil health and an obvious choice for environmental monitoring. Studies to assess the impacts of toxicity of polluted dump sites provide evidence of the potential of using earthworms via metabolomics as an indicator of ecological stress (Griffith et al., 2017). Proteomic and transcriptomic strategies have also been assessed as potential methods to monitor earthworm health. Metabolomics and proteomics approaches were used to examine such systemic impact of soil pollution. While metabolomics detected disruption in osmoregulation, proteomics results identified changes consistent with oxidative stress, apoptosis, and impeded protein synthesis (Booth et al., 2011).

Earthworms (*Eisenia fetida*) are vital members of the soil environment. Because of their

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sensitivity to many contaminants, monitoring earthworm metabolism may be a useful indicator of environmental stressors (Griffith et al., 2011). Metabolic profiles of earthworms exposed to chloroacetanilide herbicides were studied using coelomic fluid from earthworms via proton nuclear magnetic resonance spectroscopy (NMR) metabolomics and gas chromatography-mass spectrometry (GC-MS) (Griffith et al., 2017). Multiblocked-orthogonal partial least-squares-discriminant analysis (MB-OPLS-DA) and univariate analysis were used to identify metabolic perturbations in carnitine biosynthesis, carbohydrate metabolism, lipid metabolism, nitrogen metabolism, and the tricarboxylic acid cycle (Allen et al., 2015).

Intriguingly, stereospecific metabolic responses were observed between racemic metolachlor and S-metolachlor exposed worms; these support the utility of coelomic fluid in monitoring metabolic perturbations induced by chloroacetanilide herbicides in non-target organisms and reveal specificity in the metabolic impacts of herbicide analogues in earthworms (Kikuchi et al., 2018).

Metabolomics is a new experimental technique that is becoming widely used in biology, medicine and the environmental sciences for studying living organisms (Lankadurai, et al., 2014). This method measures the concentrations of the large numbers of naturally occurring small molecules (called metabolites) that are present in blood, urine, and tissues (Everts, 2018). The pattern, or fingerprint, of metabolites in the biological sample can be used to learn about the "health" of the organism (Lankadurai, et al., 2014). This approach is being used to study human diseases, with the goal to finding unique patterns of metabolites that could be used to diagnose specific diseases (Lankadurai et al., 2013).

In a similar manner, environmental metabolomics is being used to study the effects of environmental stress such as pollution and climate change on the health of fish and invertebrate organisms that live in our natural environment (Griffith, et al., 2017). Areas of application of environmental metabolomics include aquatic toxicology, terrestrial toxicology, fish diseases, aquaculture, environmental monitoring and ecological risk assessment (Lankadurai, et al., 2014). The tools used to measure metabolite levels are more commonly associated with chemistry, and include Liquid chromatography mass spectrometry (LC MS). Also, because of the immense amount of data that is collected in a typical experiment, expertise in mathematics and computer science are needed to analyze the data (Corey, et al., 2017)

Non-targeted metabolomics is a nonbiased quantitative analysis of all or a large number of metabolites found in a biological sample (Issaq et al., 2008). By contrast, targeted metabolomics analyzes a specific group of metabolites (Issaq et al., 2008; Verpoorte et al., 2008). Though metabolomics is viewed as complementary to other omic techniques it may actually provide a solution to the many shortcomings that are encountered with the other omic methods (Griffin and Bollard 2004; Bilello 2005; van Ravenzwaay, et al., 2007). Exposure of an organism to an external stressor will result in changes to gene expression and protein production, both of which are subjected to a variety of homeostatic controls and feedback mechanisms; these changes are amplified at the level of the metabolome (Nicholson et al., 1999; Ankley et al., 2006; van Ravenzwaay et al., 2007). This may result in metabolomics being a more sensitive indicator of the external stress than other omic technologies (Nicholson, et al., 1999; Ankley et al., 2006; van Ravenzwaay et al., 2007).

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Other methods have been developed to detect changes in genomic, transcriptomic, and proteomic profiles, the basic information required to make meaningful interpretations based on these data are sometimes not readily available (Ankley et al., 2006). Alternatively, the structure and function of most metabolites is fairly well characterized and may be lower in number than genes and proteins (van Ravenzwaayet al., 2007). For example, the human genome is composed of an estimated 30 000 genes, close to 100 000 proteins and about 2000 to 20 000 metabolites (Schmidt 2004; Ankley et al., 2006; van Ravenzwaay et al., 2007). As such, metabolomics studies are characterized by a higher number of samples than metabolites (variables), which allows metabolomics to be much more statistically powerful in detecting responses compared with other methods (van Ravenzwaay et al., 2012). Therefore, metabolomics has potential as a sensitive and rapid technique that in theory is capable of elucidating relationships between metabolite levels and an external stressor, whether it is contaminant exposure, nutritional deficit, or disease (Miller, 2007).

There are several challenges and difficulties in the field of environmental metabolomics, Earthworm metabolism is recognized as a useful tool for monitoring environmental pollution and measuring toxicity. Thus, the need for screening metabolites in earthworms exposed to polluted landfill dumpsites in Gombe metropolis using LC MS metabolomics.

MATERIALS AND METHODS

Study Area

The study areas are Landfill dumpsites: Inex cleaners limited dump sites opposite FCE (T), along Ashaka Road and Liji landfill dump site opposite Government girls secondary school Doma (Latitude 100 0‟N - 100 20‟N, longitude 110 01‟E - 110 19‟E). Located in Gombe city in the North Eastern region of Nigeria.

Collection of Earthworms Specimen

Earthworm specimens were collected from two different polluted landfill dump sides within Gombe Metropolis: i- Inex cleaners limited dump sites (F) ii- Liji landfill dump (L). Adult earthworms were collected from the soils at depths of 0-10 cm, 10-20 cm and 20-30 cm from the three different locations.

Treatment of the Test Organism

Earthworm were collected from the two different polluted landfill dump sites within their natural habitat which containing their food materials from their natural environment in plastic container and transported for LC MS analysis.

Metabolites Extraction from Earthworm for LC/MS Analysis

The whole Earthworms were washed with distilled water in a mortar flash-frozen in liquid nitrogen, and homogenized by bead-beating using mortar and pestle using 1 mL (50:50) cold methanol/water. The content were transferred into eppendorff tube (2ml) and homogenized using vortex for (1min), centrifuged at 1800 rpm for 20 min, and the supernatant were transferred to a new tube for cleanup with 500 µL of ice-cold chloroform. The aqueous layer were dried and stored at −4°C (Kikuchi et al., 2018).

LCMS Protocol

All samples collected were subjected to gas chromatography mass spectrometry (LC/MS) using the following protocols. Gradient elution was performed with LC-MS grade Solvent A (0.1% formic acid and 10 mMol ammonium formate in 500 mL methanol (70%) and acetonitrile (30%)) and Solvent B (0.1% formic acid and 10 mMol ammonium formate in 500 mL water) for the following gradient:

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20% A in 5 min, and 20–80% A in the next 25 min at a flow rate of 0.2 mL/min. The concentration of sample extract was 1 mg/mL and the injection volume were set to 10 µL and the UV detector was set at 210, 310, 410 and 510 nm. The MS analysis was done on Q-Exactive Focus Orbitrap LC-MS/MS system. The eluent was monitored by ESI-MS under positive and negative switching mode and scanned from m/z 100 to 1500 amu. ESI was conducted using a spray voltage of 4.2 kV. High purity nitrogen gas was used as dry gas at a sheath gas flow rate of 40 (arbitrary units) and aux gas flow rate of 10 (arbitrary units). Capillary temperature was set at 350 ◦C while aux gas heater temperature was set at 10 ◦C (Pantami et al., 2020).

LC-MS data processing

The RAW LC-MS data were processed using MZ mine software version 2.53 for LC MS based metabolomics. The following steps were used for LC-MS data processing; Raw data import; Feature detection; Chromatogram deconvolution; Isotopic peaks grouper; Peak alignment; Gap filling; Normalization; export to CSV file (Pantami et al., 2020).

RESULTS

Samples extract's Classification using PCA and PLS-DA

The most practical technique used to minimize the disparity of a multivariate data set is the unsupervised method, the principal component analysis (PCA). The PCA appears as a graphical illustration having both score Fig.1 and loading plots, which are used to detect variations or similarities among sampled data. Therefore, it can reveal the occurrence of an outlier among samples represented in a score plot. In contrast, the loading plot Fig. 2 was used to identify the potential VIP values metabolites, which are responsible for the separation observed in the score plot of the same model.

Both the score and loading plots are dependent and complementary to each other for the reasonable discussion of the discrimination among the samples. The PLS-DA loading plot Fig. 3 shows the potential marker compounds that brought the difference about the groups by PC1 and PC2, which are linear combinations of the original variables.

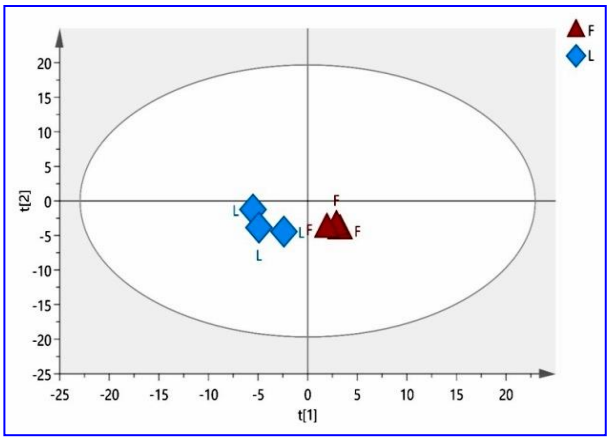

Figure 1: PCA score scatter plot showing L samples from liji landfill dumpsite and the F sample from Inex cleaner limited opposite FCE (T) Gombe. Score scatter plots which is the unsupervised model for the discrimination between the two different metabolites in earthworms from the samples L and F polluted landfill dumpsites.

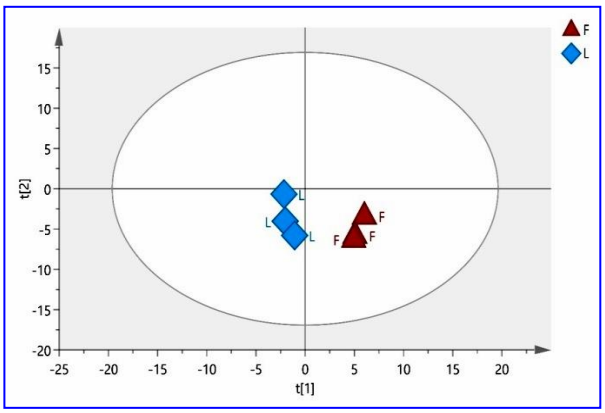

Figure 2: PLS-DA score scatter plot showing L samples from liji landfill dumpsite and the F sample from Inex cleaner limited opposite FCE (T) Gombe. The supervised model shows the variation between the samples L and F.

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Both the score and loading plots are dependent and complementary to each other for the reasonable discussion of the discrimination among the samples. The PLS-DA loading plot (Fig. 3) shows the potential marker compounds that brought about the separation of the groups by PC1 and PC2. It is observed that most of the identified compounds belong to (F) landfill dumpsite with few belonging to (L) landfill dumpsites opposite. PLS regression analysis and PLS discriminant analysis (PLS-DA) are also used often as multivariate statistical tools in metabolomics which are equally been utilized to indicate the potential marker compounds of the present study.

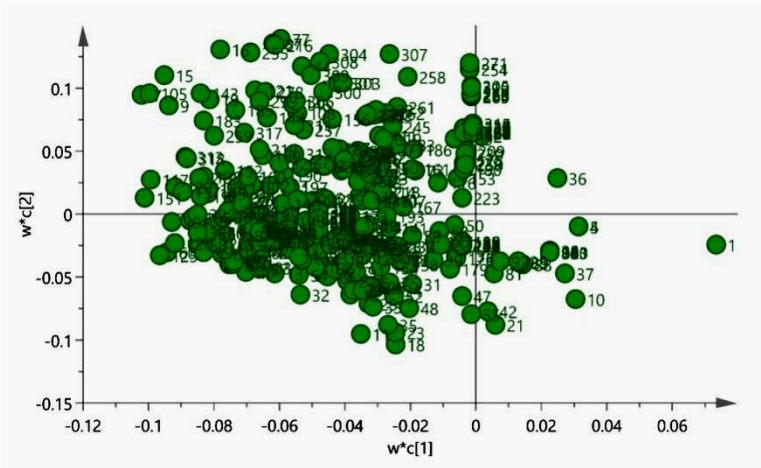

Figure 3: PLS-DA loading plot shows why the discrimination between the samples L and F.

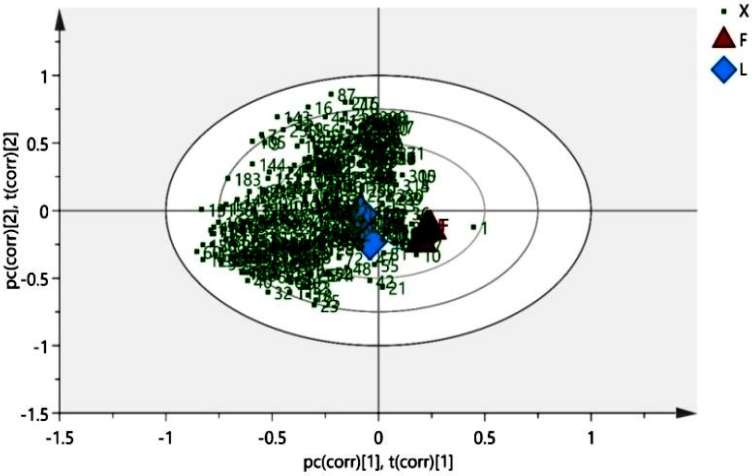

Figure 4: Biplot combine the PLS-DA loading plot and PLS-DA score scatter plot at the same plot

After VIP Plot were generated (Fig. 5), those with values greater than 1 were identified using online data base (metabolomics workbench). Table 1 shows the identified metabolites followed by their proposed structures

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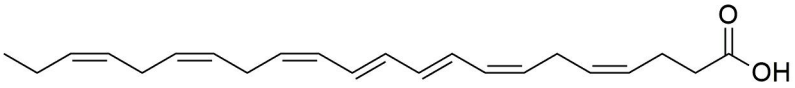
DOI: 10.56892/bima.v8i1.637

Table 1: VIP metabolites with values > 1

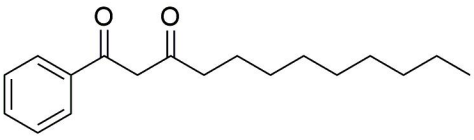
S/N	Metabolite	VIP value	*m/z*
1	4Z,7Z,9E,11E,13Z,16Z,19Z-docosaheptaenoic acid	**1.91**	327.233
2	1-Phenyl-1,3-dodecanedione	**1.85**	275.2014
3	5-(10'-Phenyldecyl)-Resorcinol	**1.79**	327.2302
4	Beta-estradiol	**1.74**	273.1859
5	1- (7Z,10Z,13Z- hexadecatrienoyl) - 3 -0- beta- D-galactosyl- sn- glycerol	**1.72**	487.2901

The VIP metabolites detected in all samples of earthworms: 4Z,7Z,9E,11E,13Z,16Z,19Z-docosaheptaenoic acid, 1-Phenyl-1,3-dodecanedione, 5-(10'-Phenyldecyl)-Resorcinol, Beta-estradiol and 1-(7Z,10Z,13Z-hexadecatrienoyl)-3-O-beta-D-galactosyl-sn-glycerol, were reported to serve as biological indicators in soil health that help in restoration of soil fertility (Kikuchi et al., 2018) and also applied as antiseptic and disinfectants (Booth et al., 2011).

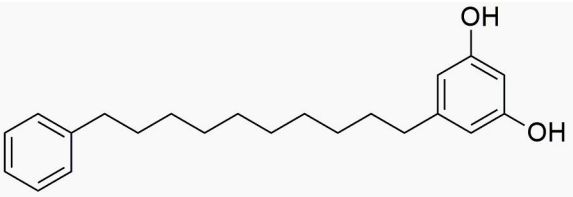
Proposed VIP Metabolites Structures

1. 

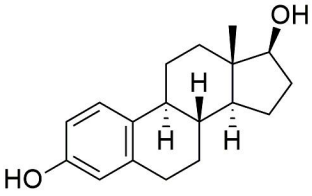
4Z,7Z,9E,11E,13Z,16Z,19Z-docosaheptaenoic acid

2. 

1-Phenyl-1,3-dodecanedione

3. 

5-(10'-Phenyldecyl)-Resorcinol

4. 

Beta-estradiol

5. 1-(7Z,10Z,13Z-hexadecatrienoyl)-3-O-beta-D-galactosyl-sn-glycero

The goodness of fit and predictability of a MVDA model are determined by calculating the R2 and Q2, which stands for fitness and predictability, respectively. Generally, R2 determines how well the training data set are statistically reproducible and when R2 intercept is less than 0.4 and Q2 intercept is less than -0.05 along Y-axis, the model is said to exhibit good fitness and predictability. The above` PLS-DA model fulfills the

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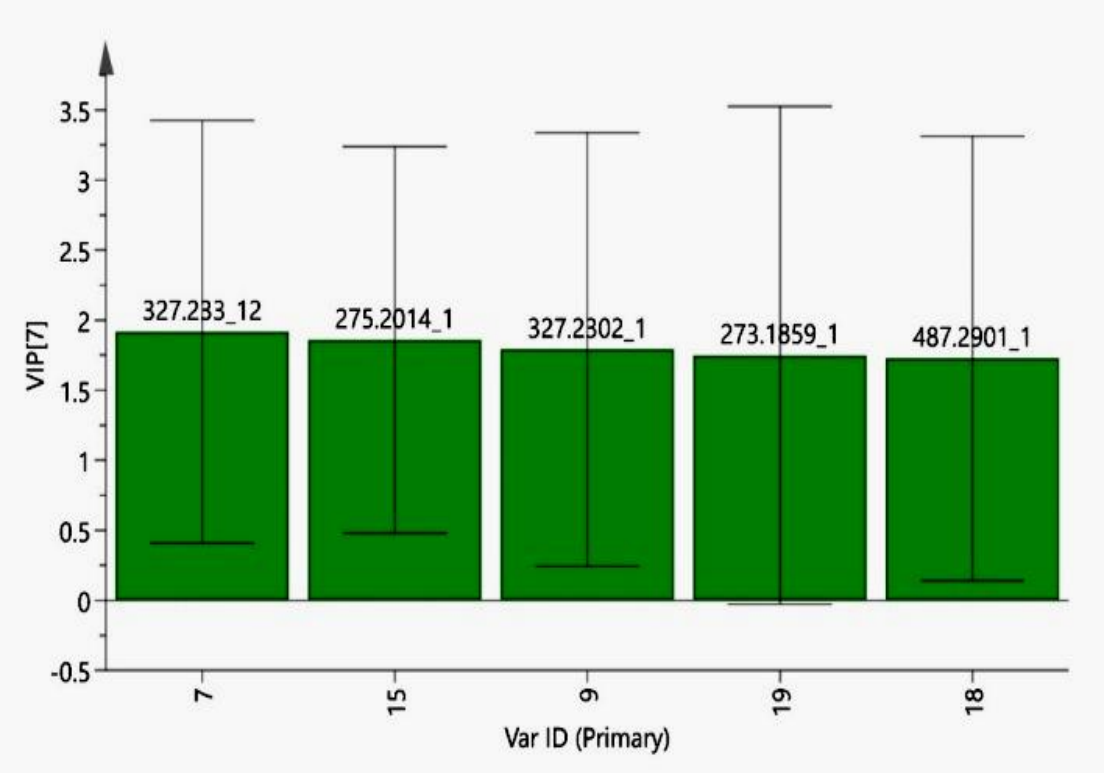
requirements for both R2 and Q2, suggesting that the model meets the criteria for the validation and prediction performance.

In the present research, the summary of fit for the score plot of the PCA suggested that the spectra of the six different replicates L and F sample were fit into the model with R2X (cummulative) as 55.6 % and a predictability value of R2Y of 91.5 % Q2 (cumulative). The model shows that the two different samples have chemical metabolites well separated by PC1 (Figure 1&2).



Fiqure 5: Vip plot shows the major compounds responsible for the variation between different metabolites extracts as listed in Table 1.

DISCUSSION

The significance of the metabolites in terms of content amounts is summed up by the variable importance for the projection (VIP) plot of the PLS-DA model (Fig. 5). The largest VIP is shown on the left side of the plot thanks to sorting. Accordingly, metabolites with VIP>1 were regarded as the highest-ranked metabolites as presented in Fig. 5. Contamination has a significant impact on earthworm biomass and density (Griffith et al., 2017). Globally, pollution of the terrestrial ecosystem is a major environmental issue. The 'earthworm acute toxicity test' has been used for many years to evaluate the possible risks that environmental toxins provide to soil invertebrates.

The outcomes of this research shows the potential marker compounds that brought about the separation of the groups (F) and (L), by PC1 and PC2. It is observed that most of the identified compounds belong to (F) Inex cleaner's limited landfill dumpsite, opppsite Federal College of education (T) Gombe with few belonging to (L) Lij landfill dumpsites opposite GGSS Doma Gombe. In

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a similar research carried out by Hamza (2021), the results indicated that six solvent extracts used for study have metabolite profiles that are clearly different from each other. The study suggested that there was a dynamic range of detectable chemical compounds in *C. vulgaris* microalga between the organic solvent extracts from the diatom. NMR- based metabolomics approach was applied to detect the variation between different solvent extracts of *C. vulgaris*. NMR spectroscopy was used to characterize the complex and metabolite-rich *E. fetida* CF, CC, and tissue metabolomes in a related study conducted by Griffith et al. (2017). The CF may act as a metabolic reservoir, according to the quantity of TCA metabolites, osmolytes, polyamines, and other metabolites found in these investigations. Our coelomocyte metabolome elucidation provides a novel means of assessing earthworm health and may provide insights on hepatocytic and immunological function, while tissue extracts provide a metabolic overview of the global events that take place within the earthworm. The study found six (6) compounds, which are consistent with the results of Corey et al. (2017), who used pooled and individual data to give a more thorough assessment of *E. fetida* endogenous metabolites. The findings of a different study by Oketola et al. (2015) on the evaluation of soil and earthworms (as bio-indicator) of heavy metals near the cattle market, Isheri, along the Lagos-Ibadan Motorway, reveal that the soil's pH was higher in every sample than it was in the control sample. (U.I. Botanical Garden) and the outcome is consistent with what Eliagu (2007) found. The research's findings also demonstrate that large concentrations of Pb, Zn, Cr are found in areas with higher pH values.

According to the summary of fit for the PCA score plot in the current study, the spectra of the six distinct replicates L: R2X (cummulative) as 0.556 R2Y 0.915. Q2 (cumulative) is 0.564. Given their greatest variability and their good separation from one another by PC, this indicates that two of the chemical metabolites were effectively isolated from the aqueous extract by PC1.

CONCLUSION

In this study, screen metabolites in earthworms exposed to polluted landfill The investigation demonstrated the putative marker molecules that caused PC1 and PC2 to separate the groups. It is noted that while a small number of the identified compounds belong to (L), the majority of them belong to (F). The VIP metabolites that were deemed to be the highest ranking ones are: 1-Phenyl-1,3-dodecanedione, 5-(10'-Phenyldecyl)-Resorcinol, beta-estradiol, 1-(7Z,10Z,13Z-hexadecatrienoyl), 4Z,7Z,9E,11E,13Z,16Z, and 19Z-docosaheptaenoic acid. Three-O-beta-D-galactosyl-sn-glycerol. The study recommends further studies to be carried out to understand and explore on improving environmental metabolomics that is being used to study the effects of environmental stress – such as pollution and climate change – on the health of fish and invertebrate organisms that live in our natural environment.

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