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Comparative Analysis of the Chemo-therapeutic Potential of *Dialium guineense* (Wild) and *Annona muricata* L. Leaf Extract on Dumpsite Leachate Induced Hepatoxicity in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author ACI designed the study, performed the statistical analysis, and wrote the first draft of the manuscript. Author SAE wrote the protocol and managed the analyses of the study. Author FUA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To compare the chemo-therapeutic potential of *Dialium guineense* and *Annona muricata* Leaf extract on dumpsite leachate induced hepatoxicity of Wister rats.
Study Design: Cross-sectional study.
Place and Duration of Study: Department of Animal and Environmental Biology (Animal Unit) and Department of Life Science, University of Benin, Benin city, Edo state, Nigeria, between January 2016 and July 2017.
Methodology: A total of 30 Wister rats were acclimatized for two weeks and randomly distributed into five groups A to E; and were administered 2ml each different treatment protocol once every 48 hours for 30days.After the exposure period, the surviving rats were examined and sacrificed. Blood

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and organs were collected for analysis. After which the organ weight indices, clinical biochemistry, tissue histology and the expression of some hepatic pro-inflammatory genes were examined.
Results: The concentration of heavy metals and anions in the test samples were above standard permissible limits. Biochemical analysis showed that leachate administration in Wister rats caused an increase in glucose 24.15%, albumin 32.73% with a decrease in alkaline phosphatase 63.91% and glucose 13.96%. Histopathological investigations indicated that the leachate provoked alterations in the liver tissue; which include mild infiltration vascular congestion, patchy vascular ulceration and a mild periportal filtrates of inflammatory cells. An increased expression of CCL11 mRNA, TGF-α mRNA and IL-1 in hepatic tissues as a result of leachate administration was observed. However, the administration of different abatements with the leachate prevented tissue damage in the Wister rat to varying degree in the following order: combined abatement >*Dialium guineense*>*Annona muricata*.
Conclusion: The findings of the present study have shown the potentials of Ikhueniro dumpsite leachate to induce tissue and genetic dysfunction probably via direct and/or indirect chemical disruption of the blood. The combined abatement was a better abatement when compared to *Dialium guineense* and *Annona muricata*.

Keywords: Chemo-therapy; Dialium guineense; Annona muricata; hepatotoxicity; Ikhueniro; leachate; Wistar rat.

1. INTRODUCTION

Indiscriminate disposal of solid waste in unauthorized places has become an increasing problem for most cities in Nigeria, especially Benin-City, Edo state. Benin-City one of the largest city in Nigeria is experiencing the problem of solid waste management despite the best attempt of waste avoidance, reduction, reuse and recovery. Use of dumpsites is still the ultimate disposal method of domestic and industrial wastes in Benin-City. This has given rise to the proliferation of open dump sites as a source of municipal solid waste disposal. Ikhueniro dumpsite is one of the most commonly used dumpsite in Benin City, Edo State; and it gets wastes through household dumps, industrial wastes, nearby markets and biological wastes.

Open dumping of solid waste poses a number of environmental threats, one of which is the production of leachates which is detrimental to both plant and animal [1,2,3]. Leachate production from dumpsite in the developing world is a big challenge. This is because most dumpsites in these regions do not have any base liner or leachate collection and treatment system; and are located in wetlands or other areas with seasonally high-water tables. The threat is further exacerbated during the wet season, when leachate spring is formed at the dumpsites. Furthermore, the topography of the land may increase the tendency of the leachate to flow towards water bodies around; putting the communities that depend on such water source at risk. It also a general notion that municipal waste dumpsite is rich in organic matter, that the dumpsite and its components are good organic manure and so has led to dumpsites being used as farm land for growing crops being ignorant of the dangers it portends. Again, increase in population has necessitated for increased in accommodation needs resulting in building houses around dumpsites without considering the health and environmental risk. Hepatoxicity is one of the most common effects associated with ingestion of toxic substances like leachate and may result to liver dysfunction and in severe cases may result to death of the organisms [4].

Dialium guineense and *Annona muricata* are gaining considerable attention in research today. Their leaves extract contains various phytochemicals which have been reported to exhibit anti-mutagenic, antiulcer, anti-neuralgic, anti-inflammatory and anti-microbial activities [5,6,7]. Administrations of the proper dosage of *Dialium guineense* and *Annona* muricata will therefore play major role in the management of various liver disorders. The leaves of *Dialium guineense* have been used for the treatment of variety of health problems, including diarrhoea, bronchitis, wound, stomach aches, malaria, jaundice, ulcer and haemorrhoids [5]. *Annona muricata* on the other hand have been reported to possess anti-neuralgic, anti-diarrheal, antidysenteric, anti-inflammatory and anti-plasmodic activities [8,9,10]. However, different natural antioxidants do not possess the same chemo-therapeutic potential to reduce the effects that leachate will induce on the organisms expose to dumpsite leachate. Some antioxidants

have more chemo-therapeutic potential than others, which go a long way in reducing drastically the effects of dumpsite leachate on the exposed organisms. The aim of this study is to compare the analysis of the chemotherapeutic potential of *Dialium guineense* and *Annona muricata* wild leaf extract on the dumpsite leachate induced hepatoxicity of Wister rats in order to ascertain the better abatement between the two studied antioxidants.

2. MATERIALS AND METHODS

2.1 Collection of Dumpsite Leachate

Raw leachate was collected from five different leachate collection points around the dumpsite and thoroughly mixed to provide a homogenous representative sample for each sampling site. This was transferred to the laboratory in pre-cleaned 4 litre plastic containers, filtered with muslin cloth to remove suspended particles and stored at 4°C until use. This was considered as the stock sample (100%) and was labelled Ikhueniro dumpsite leachate. The sample was analysed for a number of standard physical and chemical properties according to procedures outlined in the Standard Methods for the Examination of Water and Wastewater [11,12].

2.2 Collection, Identification and Classification of Plants

Fresh leaves of *Dialium guineense* (black velvet) and *Annona muricata* (Soursop) were collected from the junior staff quarters of the University of Benin and Upper Sakponba, Benin city; The taxonomic identity of the plant was confirmed at the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin, Benin City, Edo State, Nigeria. Phytochemical analysis (such as carbohydrate, alkaloids, tannin, saponin, cardiac glycoside, anthroquinone, philobotannins, steroids and flavonoid) of the crude powder of the leaves collected was determined according to the standard procedures to identify the constituents as described by [13,14,15,16,17].

2.3 Preparation of Abatements

Three different abatements were used for this study. The first abatement was the leaf extract of black velvet tree (*Dialium guineense*); the second abatement was the leaf extract of Soursop tree (*Annona muricata*); while the third abatement was combination of the leaf extract of black velvet tree (*Dialium guineense*) and Soursop tree (*Anonna muricata*) in a ratio 1:1. The leaves collected were air-dried to crispiness in the laboratory (prevailing room temperature of 30 ± 2°C) for two weeks. The dried materials were reduced to coarse form using a pestle and mortar and further pulverized to very fine particles using Viking Exclusive Joncod pulverizing machine (Model: YLH2M2 - 4). 28 g of the powdered leaves was subjected to infusion extraction and exhaustively extracted with 0.5L of warm water for Four hours. The extracts were filtered and stored at temperature of about 4°C in a clean container prior to use.

2.4 Collection and Acclimatization of Experimental Rats

Thirty (30) male and female Wister rats (6-7 weeks old) weighing within the range of 100 g to 150 g were obtained from the Anatomy Department, University of Benin, Nigeria; and housed in wooden cages with wire mesh covers. The rats were acclimatized for 2 weeks until they were 8-9 weeks and their weights taken. The animals were fed with standard rodent chow (Bendel Livestock Feeds Limited, Ewu, Edo State, Nigeria) and given distilled water ad labium.

2.5 Experimental Setup

The rats were distributed randomly into five groups of six animals (3 males and 3 females) each. The rats were administered different treatment protocol as stated below.

Group A – Control (C)
Group B - Leachate (L)
Group C - Leachate + Abatement A (LA)
Group D - Leachate + Abatement B (LB)
Group E - Leachate + Abatement C (LC)

The rats were maintained in laboratory conditions; and had access to drinking water and standard rodent chow (Bendel Livestock Feeds, Ewu, Edo State, Nigeria®) *ad libitum.* Each animal in a group was gavaged 2 ml of the different protocol as described above for 30 consecutive days (once every 48 hour). At the end of exposure period, survivors were fasted overnight, weighed (using Acculab® USA, Model-vic-303 electronic analytical weighing balance) and sacrificed under slight Anesthesia. Blood sample and the liver were collected.

2.6 Organ Weight Measurement, Collection and Preparation of Samples

Blood was collected from the inferior vena cava of the rats with plain 5ml sterilized syringe into a vial containing heparin (lithium and ammonium), fluoride oxalate (sodium fluoride and potassium oxalate) and plain bottles for biochemical analysis under a light anaesthesia. The blood in the bottles containing anticoagulants were directly centrifuged at 3000 rpm for 10 minutes to separate the plasma (supernatant); while the blood in the plain bottle was allowed to clot. Then it was centrifuged at 3000 rpm for 10 minutes to separate the serum (supernatant). The blood plasma and serum were stored at -80°C prior to biochemical analysis. The liver was surgically removed, rinsed with ice-cold physiological saline, blotted dry and weighed. The relative organ weight (organ weight/body weight × 100) was determined. While slices of the liver from exposed and control animals were fixed in 10% neutral buffered formalin before being processed.

2.7 Laboratory Analysis

2.7.1 Serum biochemical analysis

Serum biochemical markers such as total protein, albumin and alkaline phosphate were measured as functional marker for hepatotoxicity These biomarkers were determined colorimetrically by employing the standard ready-to-use kits and methods of Human. Alkaline phosphate was determined using TECO diagnostic assay kits (TECO Diagnostics, CA); total protein, albumin and glucose were determined using RANDOX assay kits (RANDOX laboratories Limited, UK). The manufacturer's instructions for each biochemical parameter were strictly followed in the course of the investigations. The absorbance of the tests was measured spectrophotometrically using OPTIMA, SP-300 (Japan).

2.7.2 Histopathological analysis of the liver

Slices of the liver from exposed and control animals were processed as described by Ibezute et al. [18]. The tissue was fixed in 10% neutral buffered formalin before being processed. All organs tissue was processed with standard protocol using Leica automated tissue processor (Model - TP1020). The organ was cut into bits and put into a tissue cassette. The cassette was label accordingly and fixed in 10% formalin solution for two hours. After fixation, the organ was dehydrated in ascending grades of alcohol (starting from 70%, 90%, 96% and absolute) for two hours each. The dehydrated organ was cleared in xylene for two hours, and impregnated in using molten wax for two hours.

Processed tissue was then embedded using Thermo scientific Histo Star embedding machine (Model - E312010). The impregnated tissue was set on a mould and placed in the hot section of the machine to dissolve the paraffin wax. Then the organ was properly set in the mould and transferred to the cold section to solidify. Sectioning was carried out using a Leica micro tomb (Model - RM 2235). The section was transferred to a Thermo scientific digital section flotation bath (Model - A82000101) to all the section spread out properly. The section was picked from the water bath using the slides and transferred to a thermo scientific slime hotplate (Model- A82100116) for the slides to dry.

The organ section was dewaxed in xylene and hydrated using ascending grades of alcohol (Absolute alcohol, 96% alcohol, 90% alcohol and 70% alcohol) to water for 3minutes in each solution. The hydrated tissue section was stained with Gill 2 haematoxylin for 10 minutes and then rinsed in water. The section was differentiated briefly with 10% acid alcohol and blue in running water for 10 minutes to develop the colour of the haematoxylin. The slide was counter stained with Shandon Eosin and rinsed in water afterwards. The stained slides were again dehydrated using ascending grades of alcohol (starting from 70%, 90%, 96% and absolute); and cleared in xylene for 5 minutes. The section was mounted using shandom's mount (Distrene Dibutyl Phathalate xylene), covered with a cover slip and allowed to dry. The slides were examined Leica CME light microscope (Model – 1349522X).

2.7.3 Expression of hepatic pro-inflammatory genes

The levels of expression of certain hepatic pro-inflammatory genes were assessed using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) techniques. In brief, RNA from the liver samples was extracted using the spin column kit obtained from Aidlab's EASYspin PlusVR (Aidlab Biotechnologies Co., Ltd, Beijing, China) according to the instructions of the manufacturer. The RT-PCR was carried out with 500 ng RNA template using the Transgen EasyScriptVR one-step RT-PCR

Table 1. Sequences of gene-specific primers

S/N	Gene	Sequence (50–30)
1.	B-Actin	Forward: GTCAGGTCATCACTATCGGCAAT
		Reverse: AGAGGTCTTTACGGATGTCAACGT
2.	TGFβ	Forward: GCTGAACCAAGGAGACGGAA
		Reverse: CCACGTAGTAGACGATGGGC
3.	CCL11	Forward: CACCCAGGTTCCATCCCAAC
		Reverse: GGGGATGGGTGCCGATATTC
4.	IL1α	Forward: CCATCCAACCCAGATCAGCA
		Reverse: TCTCCTCCCGATGAGTAGGC

supermix (Beijing TransGen Biotech Co., Ltd, Beijing, China) according to the instructions of the manufacturer. Samples were subjected to an initial incubation at 45°C for 30 min for cDNA synthesis, followed by PCR amplification, using gene-specific primers (GSP) (Table 1), 94°C for 5 min followed by 50 cycles of 94°C for 30s, 5 min at the annealing temperature of GSP, and 1 min at 72°C. All amplifications were carried out in C1000 Touch^{TM} Thermal Cycler (BioRad, Hercules, CA). The intensity of the amplicon bands on 1.5% agarose was analysed using Image J software [17]. Results were presented as the relative expression of the gene in comparison with the level of expression of B-Actin gene.

2.8 Data Analysis

All statistical analyses were conducted with Statistical Package for Social Scientists (SPSS) and Microsoft Excel computer software. Data are presents as mean ± Standard Error (SE). One-way Analysis of Variance (ANOVA) was used to determine the differences among various groups.

3. RESULTS AND DISCUSSION

The relative liver weight of Wister rats are shown in Fig. 1; it was observed that leachate administration caused an increase in the relative weight of the liver (4.10%) of Wister rats when compared to the control (3.31%). This increase in weight may be as a result of increased in metabolic activities of the liver as a result of the presence of unwanted substances in the system of the wistar rats. A similar finding has earlier been reported by Alimba et al. [4] who reported an increase absolute and relative liver of wistar rats. This according to Alimba et al. [4] is as a result of leachate from Olusosun and Aba-Eku landfills in South-western Nigeria. The relative liver weight of rats administered leachate with different abatement protocols decreased except in rats administered with *Annona muricata* abatement when compared to the relative liver weight of leachate administered rats.

The result for the biochemical analysis of the Wister rats is shown in Table 2. It was noted that the administration of leachate only significantly increased the serum concentration of alkaline phosphatase (ALP) and total protein; with a decrease in serum albumin in the rats when compared to the control. The co-administration of *Annona muricata* and *Dialium guineense* with leachate caused a reversal in the trend observed in leachate treated rats. This study showed that leave extract of *Annona muricata* and *Dialium guineense* restored the biochemicql profile of the liver which correlates with the histopathological architecture of the liver. This improvement may be as a result of the anti-oxidant capacity of the leaves extract. This is in agreement with the findings of Azab [19] who found out that elevation in ALP and total protein with reduced albumin induced by lead was reversed by sesame oil. Baxla et al. [20] and Metwally and Hashem [21] also noted that the elevation in ALP as a result of lead and cadmium respectively was reversed by the administration of *Curcuma longa* and garlic respectively.

The relative expression of genes is shown in Fig. 2. It was noted that the administration of only leachate (0.56) caused an increase in the relative expression of hepatic Eotaxin (CCL_{12}) Interleukin 4 (IL-4) and Transforming growth Factor (TGF) gene in Wister rats, when compared to the control (0.48). Co-administration of *Dialium guineense* abatement and *Annona muricata* abatement along with leachate caused a further increase in the relative expression of hepatic CCL_{12} gene (0.91 and 1.19 respectively). The co-administration of *Dialium guineense* abatement and *Annona muricata* abatement along with leachate caused a reversal in the trend of expression of IL-A and TGF gene when compared to the leachate administered groups of rats. Similar trend has been reported by Soliman et al. [22] who reported that Curcumin

administration decreased the expression of IL-1 and IL-8 expression in the liver of wistar rats previously increased by paracetamol administration.

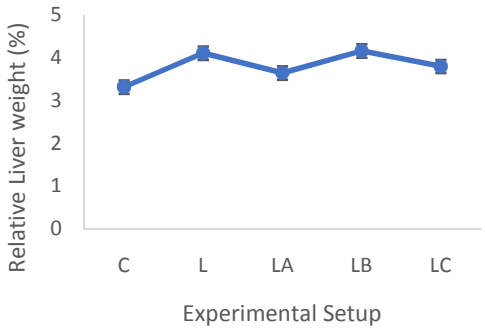

Fig. 1. Relative liver weight of Wister rats administered Ikhueniro dumpsite leachate and various abatements

Plate 1 to 5 shows the histological findings in the hepatic tissues of the wistar rats exposed to the protocols. Haemato-xylene and Eosin stained hepatic tissue micrographs revealed that the tissues lined up in order, and transverse striation showed that the hepatocytes, sinusoids, bile ducts and hepatic artery were clear and well distributed in the control group (Plate 1). However, leachate administration caused a patchy vascular ulceration, mild vascular congestion and mild periportal infiltrates of inflammatory cells (Plate 2). The histological alteration may be associated with corrosive nature of Ikhueniro dumpsite leachate. Similarly, Alimba et al. [4] reported a mild to severe multifocal degenerative and necrotizing hepatitis which is shown by the presence of a diffused hydropic degeneration of hepatocytes and multiple foci of periportal zones; shrunken hepatocytes and diffused hepatic necrosis with cellular infiltration by macrophages and lymphocytes as a result of Olusosun and Aba-Eku landfills in South-western Nigeria.

The co-administration of leachate with *D. guinensis* a recovery from the histological alterations previously noted with the presence of mild kupffer cell activation (Plate 3 and 5). Metwally and Hashem [21] also reported that alterations that associated with Cd-toxicity such as enlarged hepatocytes, with severe hydropic degeneration and coagulative necrosis were significantly alleviated by garlic administration. In plate 4, mild kupffer cell activation was also observed in the liver of wistat rats co-administered leachate with *A. muricata* along with mild periportal infiltrates of lymphocytes and mild vascular congestion.

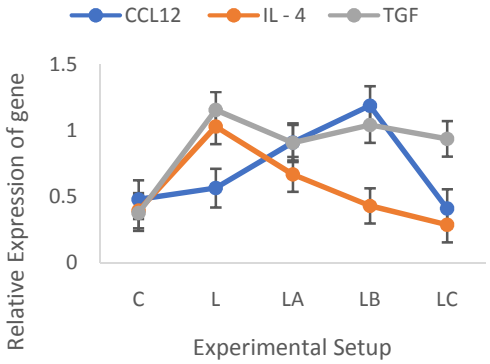

Fig. 2. Relative expression of hepatic pro-inflammatory gene of Wister rats administered Ikhueniro dumpsite leachate and various abatement

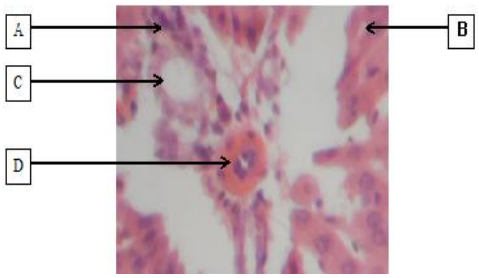

Plate 1. Photomicrograph of Wister rat liver in the control group (A, hepatocytes, B, sinusoids, C, bile ducts and D, hepatic artery) (H&E x 100)

Table 2. Some liver function parameters of Wister rats administered Ikhueniro dumpsite leachate and various abatements

	C	L	LA	LB	LC
Albumin (mg/dl)	2.75±0.05	2.65±0.23	3.38±0.18	3.44±0.11	4.08±0.13
Alkaline phosphatase (mg/dl)	40.92±8.85	47.71±5.86	21.10±4.40	44.08±6.38	30.25±14.65
Total protein (mg/dl)	7.18±0.33	8.16±0.63	7.05±0.20	7.25±0.50	7.90±0.40

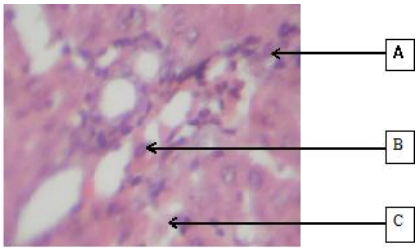

Plate 2. Photomicrograph of Wister rat liver exposed to leachate only (A, mild periportal infiltrates of inflammatory cells, B, patchy vascular ulceration and C, mild vascular congestion) (H&E x 100)

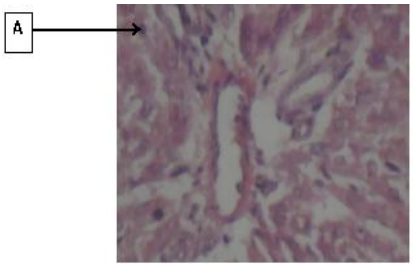

Plate 3. Photomicrograph of Wister rat liver exposed to leachate +*D. guinensis* (A, mild kupffer cell activation) (H&E x 100)

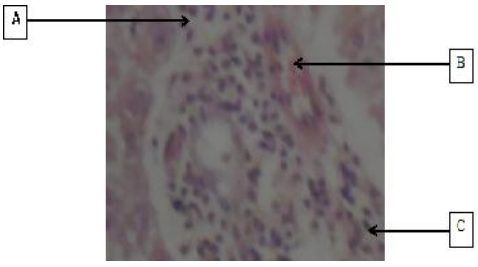

Plate 4. Photomicrograph of Wister rat liver exposed to leachate + *A. muricata* A, mild periportal infiltrates of lymphocytes, B, mild vascular congestion and C, mild kupffer cell activation (H&E x 100)

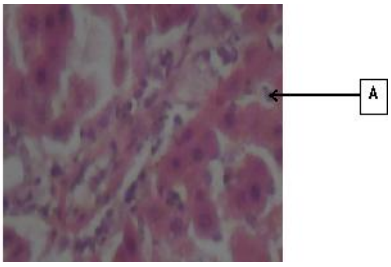

Plate 5. Photomicrograph of Wister rat liver administration leachate + combined extract (A, mild kupffer cell activation) (H&E x 100)

4. CONCLUSION

This study shows that the *Dialium guineense* and *Annona muricata* leaves are rich in phytochemicals and antioxidants. The utilization of these plants help prevented toxicity associated with dumpsite leachate in the following order, combined abatement > *Dialium guineense* > *Annona muricata*. The combined abatement was a better abatement when compared to *Dialium guineense* and *Annona muricata*.

ETHICAL APPROVAL

This research design was reviewed and approved by the Faculty of Life Science Ethical board, University of Benin (FLS/REC/2015/056).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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