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Comparative Chelating and Ameliorative Effects of *Bryophyllum pinnatum* Ethanolic Leaf Extract and Succimer on Gonadotropin Hormone of Male Wistar Rats Exposed to Methylmercury

[1]*Onyagbodor, O. P., [2]Aprioku J. S., [1]Vincent-Akpu I. F.

[1]Department of Animal and Environmental Biology, Faculty of Science, University of Port Harcourt, P. M. B 5323, Choba. Port Harcourt, Nigeria.

[2]Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt, P. M. B 5323, Choba. Port Harcourt, Nigeria.

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Abstract: **Methylmercury exposure and intoxication in human and animal populations has increased in recent years due to exposure from various sources. Methylmercury has been implicated to be the number one hormonal disruptor. This study explores the comparative chelating effects of *Bryophyllum pinnatum* ethanolic leaf extract from a plant that is largely used in folkloric medicine to treat various ailment and succimer, a standard agent used for the treatment of mercury intoxication, on testosterone and the gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) of Wistar rats. Fresh *Bryophyllum pinnatum* leaves were harvested, air dried, pulverized and active components extracted using 70% ethanol. The hormonal analysis was carried out using enzyme-linked immunosorbent assay (ELISA) kits. The median lethal dose (LD_{50}) of methylmercury was 7.07 mg/kg and was determined using Lorke's method. The animals were randomly group in eight (8) groups, the Control (Distilled water); Methylmercury (MetHg) 1mg/kg; MetHg (1mg/kg) + BPELE (100mg/kg); metHg (1mg/kg) + BPELE (200mg/kg); BPELE (100mg/kg); BPELE (200mg/kg); metHg (1mg/kg) + succimer and succimer alone. The results showed that for testosterone, MetHg (1mg/kg/bw) combined with *B. pinnatum* (100 mg/kg) and MetHg (1mg/kg/bw) combined with Succimer (5mg/kg/bw) were significant $p<0.001$ compared to the control. Principal Component Analysis and Dendrogram were used for the analysis. Testosterone decreased significantly $p<0.001$ compared to the control. There was an ameliorative increase in testosterone production in the combination groups of MetHg(1mg/kg/bw) +*B. pinnatum* (100mg/kg/bw) and MetHg(1mg/kg/bw) + succimer (5mg/kg/bw) compared to MetHg alone group. However, succimer showed a better ameliorative increase in testosterone than *B. pinnatum*. The results for FSH for both *B. pinnatum* 100 and 200 mg/kg/bw were significant compared to the control, there was also significant difference in the succimer and MetHg combined groups but at different level of significance. There was no statistical difference in the LH results amongst the treatment groups compared to the control. This study suggests that *B. pinnatum* has ameliorative effects against methyl mercury. as observed in the improvement of testosterone production in the testis, otherwise suppressed by methylmercury administered orally to Wistar rats. BPELE could be an ameliorative blocker for the disruptive effects of methylmercury on male reproductive hormones and that BPELE could be a possible herbal chelate for methylmercury poisoning.**

Keywords: **Methylmercury, Succimer, *Bryophyllum pinnatum*, Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone.**

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1. INTRODUCTION

Mercury intoxication has implicating effects on male reproductive function, plethora of data exists on its adverse effects on spermatogenesis and hormonal disruption (Wagner *et al*., 2017).The mammalian male reproductive function can be affected through a direct effect of the testes and other tissues of the reproductive organ, causing decreased or altered sperm production, through impairment of the accessory sex gland secretions, and/or indirectly through the neuroendocrine system, resulting in hormonal imbalance. Adverse effects on male fertility include altered spermatogenesis, genetic disease in offspring (Nordberg, Fowler, & Nordberg, 2014). Common end-points for assessment of male reproductive function include size of testis, weight and volume, semen quality, secretory function of prostate and seminal vesicles, reproductive endocrine function, impotence or reduced libido and fertility. Organic mercury compounds are classified into three types: aryl ring, short chain alkyl, and long chain alkyl. While the aryl and long-chain forms of mercury rapidly convert to inorganic mercury, methylmercury has a short-chained form that is rapidly and almost completely absorbed from the gastrointestinal tract and can be distributed throughout the body while remaining in their organic forms (Broussard, Hammett-Stabler, Winecker, & Ropero-Miller, 2002; Erdemli-Köse, Yirün, Balci-Özyurt, & Erkekoğlu, 2022).

Rats exposed to methylmercury were observed to have significant reduction in intra-testicular testosterone and somewhat lowered epididymal sperm count were found in those treated with high-dose, whereas inverse correlation was observed between fertility and testicular mercury content. Also significant positive correlation between serum total testosterone, but not free testosterone (Agrawal & Chansouria, 1989), and cumulative mercury exposure was found in workers exposed to mercury vapour. Mercury exposure has also been shown to induced sperm DNA damage and abnormal sperm morphology and motility (Balali-Mood, Naseri, Tahergorabi, Khazdair, & Sadeghi, 2021; Barregård, Lindstedt, Schütz, & Sällsten, 1994; Friedmann, Chen, Rabuck, & Zirkin, 1998; Henriques, Loureiro, Fardilha, & Herdeiro, 2019; Lee, Kim, & Ryu, 2019). Methylmercury is a highly neurotoxic, short-chained alkyl mercury compound that poses a high risk of exposure to humans and animals through consumption of contaminated fish and seafood.

Inorganic mercury enters the food chain in the aquatic environment and is converted to methylmercury by sulphate-reducing bacteria. Organic mercury compounds bioaccumulate and reach toxic levels in large fish.

Bryophllum pinnatum is known as the wonder herb that is used to treat a variety of ailment and it is globally used in folkloric medicine (García-Pérez *et al.*, 2021). The plant, also known as *Kalanchoe pinnata*, has not been explored to potentially ameliorate methylmercury disruptive effects on testosterone, follicle stimulating hormone and luteinizing hormones. mercury poisoning of the reproductive tract. With low doses of 120mg/kg/bw, there were neither observed testicular, sperm nor hormonal toxicity. Although many herbal remedies' folklore claims have yet to be scientifically validated, B. pinnatum has been fairly studied, with most of the claims justified. This has increased the promotion of B. pinnatum and other plants as alternatives or supplements to conventional medicines. Furthermore, the high cost of orthodox medications, as well as the development of resistance to most orthodox chemotherapeutic agents, has led to the use of herbal medications such as B. pinnatum leaf as alternatives, particularly in developing countries (Aprioku & Igbe, 2017; Osujih, 1993; Saad, Azaizeh, Abu-Hijleh, & Said, 2006). With the exception of calcium carbonate, chemomicroscopic analysis of Bryophyllum pinnatum revealed the presence of cellulose, tannins, starch, lignin, calcium oxalate, suberin, aleurone grain, and mucilage. Both aqueous and methanolic extracts contained phytochemicals such as alkaloids, phenols, flavonoids, saponins, tannins, carbohydrates, and triterpenes but not anthraquinones. A study has also suggested that the leaves of B. pinnatum have mild antioxidant potential (Namadina et al., 2020). Traditional medicines and food both use the leaves of Bryophyllum pinnatum (Lam.) Oken and the potentials of the aqueous extract and fractions of B. pinnatum leaves' antioxidant activity (reducing power, DPPH, ABTS, FRAP, H2O2 scavenging ability, and metal ion chelating), carbohydrate-digesting enzyme activity, and inhibitory activity of cholinergic enzyme have been studied (Ojo et al., 2018).

Chelators such as dimercaprol, succimer are used for the treatment of heavy metals poisoning, including mercury intoxication. While dimercaprol has been found to redistribute mercury to other soft tissues (Katzung, Masters, & Trevor, 2012), succimer and unithiol are reputed to be effective for the expulsion of mercury after intoxication. These drugs may be effective but there are no evidences that they completely address the damaging effects on the male reproductive and endocrine function of mammals (Adams, Frederick, Larkin, & Guillette Jr, 2009; Hintelmann, 2010; Jayasena, Frederick, & Larkin, 2011; Zimmermann et al., 2013). Succimer chelation for low level organic mercury exposure in children has

limited efficacy (Cao et al., 2011). Although chelating agents administered for chronic intoxication may accelerate the excretion of heavy metals, their therapeutic efficacy in terms of decreased morbidity and mortality is largely unestablished. Recent investigations suggest that their use in such settings might be associated with deleterious effects. Potent mercury chelators, such as unithiol and succimer predominantly remove mercury from the kidney. Experiments have shown that they are inefficient in reducing the mercury content (Kosnett, 2010). The aim of this study is to determine the ameliorative effects of B. pinnatum ethanolic leaf extract on the disruptive effects of methylmercury on the reproductive hormones of male Wistar rats.

2. MATERIALS AND METHODS

Plant material

Fresh leaves of B. pinnatum were collected from the botanical garden of the University of Port Harcourt, Port Harcourt. The leaves were washed and air dried, the dried powder were weighed and then extracted by maceration in hydroethanolic (70% Ethanol) medium at room temperature The extract was decanted and filtered using cotton handkerchief in a funnel and further filtered using Whatman No. 1 quantitative circle filter paper of 24.0 cm with cat. No. 1001 240. The filtrate was macerated twice using the same volume of solvent to exhaustively extract the leaves. The ethanol was then removed from the extract by evaporation under reduced pressure using a Rotary Evaporator RE-52A, E. Track Instruments England at 53°C to a constant volume. The extract was preserved in a desiccator. Water was used as diluent for the formulation of the doses (Dada & Ojo, 2018; Faleye & Dada, 2016).

Experimental animals and housing

Sixty-four (64) male Wistar rats of average weight of 201g, obtained from Priceless test animal farm, Badagary, Lagos, Nigeria, were used for the study. The animals were acclimatized for two weeks in the Animal house of Department of Animal and Environmental Biology, University of Port Harcourt and were given standard rodent feed and clean water ad libitum. The animals were kept in a well-ventilated room with a 12 hours light and dark cycle at room temperature. All animal were anesthetized using diethyl ether experimental procedures were approved by the Animal Research Ethics Committee of the University, in accordance to the guide for care and use of laboratory animals (Rowsell, 1991)

Chemicals and Drugs

Methylmercury (CH_3Hg) and Succimer (meso-2,3-Dimercaptosuccinic acid) were both purchased from Sigma-Aldrich, Germany. A Sigma Due Diligence form was completed with a customer declaration of specific uses of controlled and voluntary monitored substances before these chemicals were shipped.

Preliminary Study

Acute toxicity test (LD_{50}) was carried on the plant, *Bryophyllum pinnatum* Ethanol leaf extract and methylmercury (Lorke, 1983).

Experimental Design

Rats were divided randomly into eight groups as follows:

1) **Control group (n = 8):** Adult male rats administered distilled water daily by gavage for 42 days.

2) **Methylmercury (MetHg) alone –** 1mg.kg/bw was administered to the rats for 42 days

3) **Methylmercury + B. pinnatum (100mg/kg/bw) –** this group was first administered with MetHg for 14 days and then given the B. pinnatum leaf extract was administered together with mercury for another 28 days, making a total of 42 days.

4) **Methylmercury + B. pinnatum (200mg/kg/bw) –** this group was first administered with MetHg for 14 days and then given the B. pinnatum leaf extract was administered together with mercury for another 28 days, making a total of 42 days.

5) **Methylmercury + succimer (5mg/kg/bw) –** this group was first administered with MetHg for 14 days and then given the succimer for another 28 days, making a total of 42 days.

6) **B. pinnatum (100mg/kg/bw) –** this group was first administered with *B. pinnatum* leaf extract for 42 days.

7) **B. pinnatum (100mg/kg/bw) –** this group was first administered with *B. pinnatum* leaf extract for 42 days.

8) **Succimer (5mg/kg/bw) –** this group was first administered with *B. pinnatum* leaf extract for 42 days.

Statistical analyses

Statistical analyses were performed by the standard method. All the results were expressed as mean ± standard deviation (S.D.). The mean of the all groups compared using One - way ANOVA by SPSS (Statistical Package for Social Sciences) and Tukey's post-hoc test. P -value of less than 0.05 was considered to represent statistically significant change (Abo-Allam, 2003; Levesque, 2005). Multivariate analysis from Minitab was used, showing cluster and dendrogram of the results.

3. RESULTS AND DISCUSSION

Effect on Testosterone

Table 1 shows (Broussard et al., 2002)MetHg (1mg/kg) group was dosed with methyl mercury for 42 days and showed depleted levels of testosterone with a mean value of 0.02 ± 0.01 ng/ml. This was significantly lower than values in groups down the table at 95% confidence interval.

BPELE groups were dosed 100mg and 200mg of the extract respectively for 42 days and showed normal testosterone levels which did not significantly differ ($p < 0.05$), from the control . This also did not significantly differ from succimer alone group which was dosed with succimer for 42 days but showed higher testosterone levels at 1.85 ± 0.69 ng/ml.

The ameliorative effects of MetHg (1mg/kg) + BPELE (100mg/kg & 200mg/kg) and MetHg(1mg/kg) + succimer groups were compared in groups 7, 8 and 11. Methylmercury was administered to the three experimental groups for an initial 14 days, then 100mg extract, 200mg extract and succimer (5mg/kg) was administered to the combination groups for a further 28 days, until the 42nd day. BPELE (200mg/kg) showed the highest ameliorative effect with testosterone values of 1.91 ± 0.22ng/ml. This was followed by group MetHg (1mg/kg) + succimer (5mg/kg) with a value of 1.25 ± 0.11ng/ml which was significantly lower than group, BPELE (200mg/kg). MetHg (1mg/kg) + BPELE (100mg) had the lowest testosterone values at 0.3 ± 0.06 ng/ml indicating the ameliorative effect among the treatment groups.(Barkallah et al., 2020)

Effects on Follicle Stimulating Hormonal

Follicle stimulating hormone levels were highest in the control group with levels at 1.45 ± 0.95 ng/ml. This was significantly higher ($p < 0.05$) than all other test groups.

BPELE (100mg/kg & 200mg/kg) groups were administered 100 and 200 mg/kg of the extract only, respectively and they also showed relatively high levels of FSH at 0.44 ± 0.22 IU/L and 0.53 ± 0.4 IU/L but did not significantly differ from the other groups at 95 per cent confidence interval level. Succimer only group was administered and presented lower FSH levels at 0.15 ± 0.1 IU/L but was not significantly different at $p < 0.05$.

MetHg (1mg/kg) + BPELE (100mg/kg), MetHg (1mg/kg) + BPELE (200mg/kg) and MetHg (1mg/kg) + succimer (5mg/kg) were considered for the ameliorative effects of the 100mg, 200mg extracts and succimer respectively. The results across the groups did not show a significant level difference in their values a $p < 0.005$.

Effects on Luteinising

MetHg (1mg/kg) + BPELE (100mg/kg) , MetHg (1mg/kg) + BPELE (200mg/kg) and MetHg (1mg/kg) + succimer (5mg/kg) were considered for the ameliorative effects of the 100mg, 200mg extracts and DMSA respectively. The results across the groups did not show a significant level difference in their values a $p < 0.005$. MetHg (1mg/kg + BPELE (100mg extract), however showed a higher LH level (0.65 ± 0.35mlU/L) than MetHg (1mg/kg + BPELE (100mg extract), (0.29 ± 0.06mlU/L) and 12 (0.39 ± 0.11mlU/L).

Control, succimer, BPELE (100mg/kg) and BPELE (200mg/kg) were considered for the effects of water, succimer, BPELE (100mg and 200mg) of the extracts respectively. BPELE (100mg) showed a significantly higher LH levels (2.18 ± 0.4mlU/L) than other groups. BPELE showed the lowest LH levels with value at 1.04 ± 0.47 mlU/L.

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Table 1: Ameliorative Effects on Hormonal levels of Male Wistar Rats exposed to Methyl Mercury and Treated with succimer and BPELE

Group Names	Testosterone, ng/ml	FSH, IU/L	LH, mlU/L
Control Distilled Water	2.13 ± 0.36	1.45 ± 0.47	1.34 ± 0.71
MetHg (1mg/kg)	0.02 ± 0.003^{d}	0.32 ± 0.17	0.82 ± 0.61
MetHg (1mg/kg) + BPELE (100mg/kg)	0.3 ± 0.06$^{\delta}$	0.42 ± 0.02 ^{c}	0.65 ± 0.17
MetHg (1mg/kg) + BPELE (200mg/kg)	1.91 ± 0.11	0.38 ± 0.09 ^{b}	0.29 ± 0.03
MetHg (1mg/kg) + Succimer (5mg/kg)	1.25 ± 0.95^{b}	0.44 ± 0.05	0.34 ± 0.02
BPELE (100mg/kg)	1.43 ± 0.05 $^{\delta}$	0.44 ± 0.10 ^{b}	2.18 ± 0.19
BPELE (200mg/kg)	1.71 ± 0.17$^{d\,\delta}$	0.53 ± 0.19 ^{b}	1.04 ± 0.23
Succimer (5mg/kg)	1.85 ± 0.34$^{d\,\delta}$	0.15 ± 0.05	1.37 ± 0.60

n = 8 animal per group

Values are expressed as mean ± SEM

Value significant at P<0.05 compared to the control

γ - the mean difference is significant at the p<0.001 compared with metHg alone group

δ – the mean difference is significant at the p<0.0001 compared metHg alone group

b - the mean difference is significant at the p<0.01 compared control

c - the mean difference is significant at the p <0.001 compared control

d - the mean difference is significant at the p<0.0001 compared control

MetHg – Methylmercury

Succimer - (meso-2,3-Dimercaptosuccinic acid)

BPELE – *Bryophyllum pinnatum* ethanolic leaf extract

For the combination groups, metHg was administered for 14days alone first, followed by BPELE and succimer for another 28 days till the 42nd day,

To better understand the effects of the various treatments, multivariate analysis techniques utilizing principal component analysis were used. Full spectrum hormone profiling has the potential to assay more variables per sample than is typically done.A mathematical algorithm called principal component analysis (PCA) reduces the number of dimensions in the data set while preserving the majority of the inherent variation (Jolliffe, 2002). This is accomplished by locating the principal components—or directions—along which the data's variation is greatest. Each sample can be represented by relatively few numbers rather than values for numerous variables by using a small number of components (Ringnér, 2008). PCA can be a useful technique to aid comprehension of hormone profiling experiments because it identifies new variables, the principal components, which are linear combinations of the original variables. 2010's Albacete, Ghanem, Dodd, and Pérez-Alfocea

Principal component analysis of hormone profiling data suggests an important role for of B. pinnatum countering the effects of methylmercury in Wistar rat testosterone levels.

Principal component analysis including 8 groups treated in combination or alone with B. pinnatum, succimer of mercury identified a principal component mainly unifying the variance of the testosterone, follicle stimulating hormone and Luteinizing hormones. principal component analysis including the threes extracted the principal component.

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Testosterone ng/ml

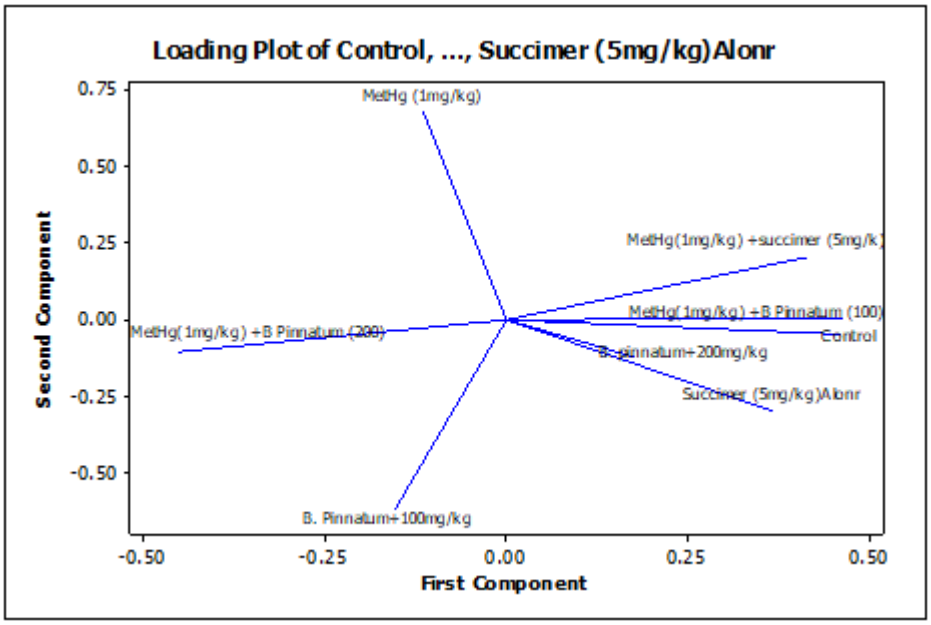
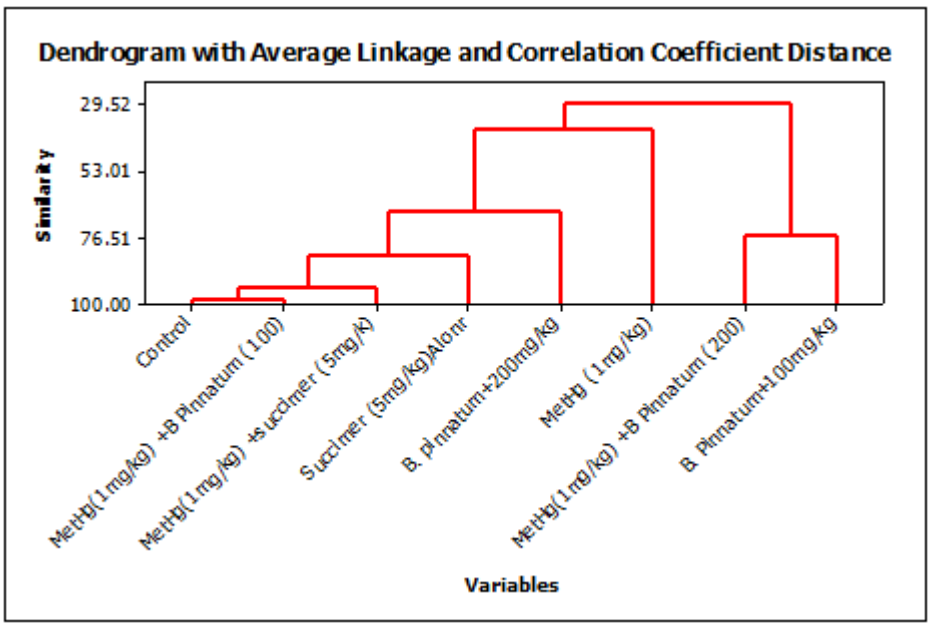


Figure 1 Two axes of a principal components (FC, SC) analysis showing levels of testosterone after treatment with b. pinnatum alone and in combination with MetHg and succimer combination or alone. by arrows) and the position of various hormonal and ionic variables (denoted by abbreviationsThe circles enclose those variables that fall into the same cluster (95% confidence level).

Step	clusters	level	level	joined	cluster	cluster
1	7	98.3614	0.03277	1 3	1	2
2	6	94.0401	0.11920	1 5	1	3
3	5	83.0626	0.33875	1 8	1	4
4	4	75.7820	0.48436	4 6	4	2
5	3	67.1457	0.65709	1 7	1	5
6	2	38.7562	1.22488	1 2	1	6
7	1	29.5194	1.40961	1 4	1	8



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The dendrogram for testosterone shows the control and the MetHg (1mg/kg) + BPELE(100mg/kg) are more closely similar in the cluster and at the next level the metHg (1mg/kg) + succimer (5mg/kg) shows similarity with the two groups with slightly higher variability. Furthermore, the succimer alone group shows similarity with the three other groups mentioned above but at a higher and different level. BPELE (200mg/kg) has another level of similarity its chunk connects to the clades that links other groups at a lower level. MetHg (1mg/kg) is the highest clade with the longest leaf in the group clusters with a wider viability from the others. There are seven clusters in all, five of which are grouped together and two together. MetHg (1mg/kg) + BPELE (200mg/kg) and BPELE (100mg/kg) shows with each other different from the other clusters.

Follicle stimulating hormone IU/L

Control	0.312	-0.467	0.160
MetHg (1mg/kg)	0.431	-0.307	0.241
MetHg(1mg/kg) +B Pinnatum (100)	0.370	0.205	-0.488
MetHg(1mg/kg) +B Pinnatum (200)	-0.467	-0.299	0.046
MetHg(1mg/kg) +succimer (5mg/k)	-0.481	-0.267	0.078
B. Pinnatum+100mg/kg	-0.034	-0.144	-0.733
B. pinnatum+200mg/kg	0.319	-0.478	-0.002
Succimer (5mg/kg)Alone	-0.168	-0.485	-0.363

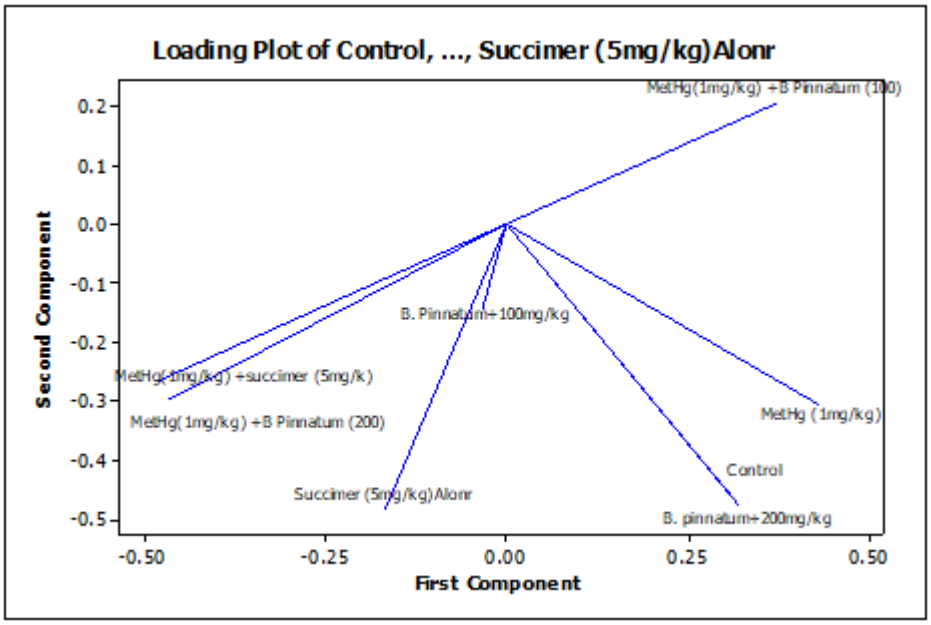

Figure 1: Principal Component Analysis (PCA) of Follicle stimulating hormone (IU/L)

Step	clusters	level	level	joined	cluster	cluster
1	7	99.8630	0.00274	4	5	4	2
2	6	98.8386	0.02323	1	7	1	2
3	5	95.4659	0.09068	1	2	1	3
4	4	84.1872	0.31626	6	8	6	2
5	3	67.9518	0.64096	4	6	4	4
6	2	54.3458	0.91308	1	3	1	4
7	1	44.1907	1.11619	1	4	1	8

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Dendrogram

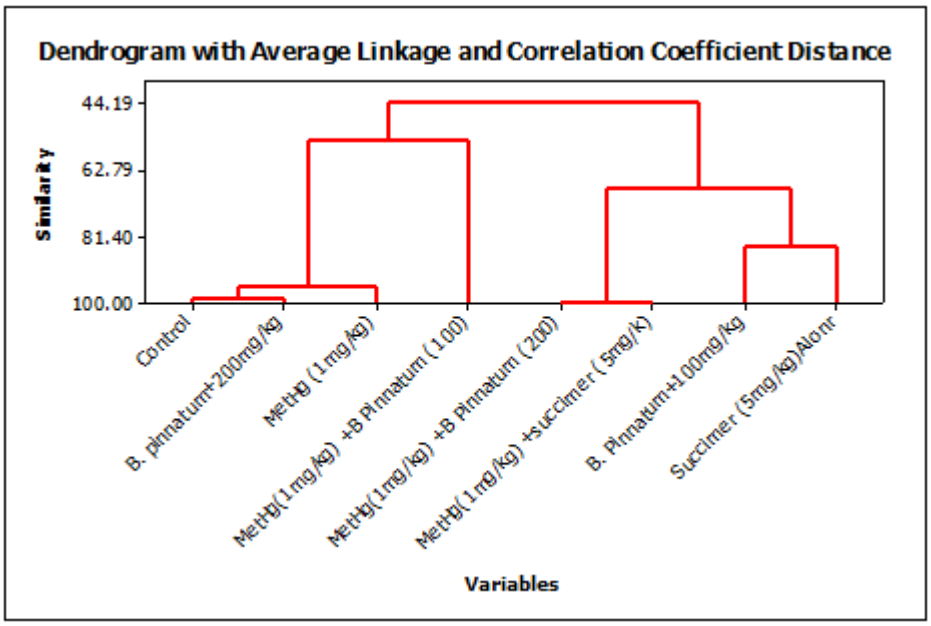

Figure 2: Dendrogram plot for Follicle stimulating hormone (IU/L)

Luteininizing hormone mlU/L

Control	0.073	0.555	0.349
MetHg (1mg/kg)	-0.420	-0.355	0.064
MetHg(1mg/kg) +B Pinnatum (100)	-0.239	-0.057	-0.686
MetHg(1mg/kg) +B Pinnatum (200)	-0.384	0.331	-0.310
MetHg(1mg/kg) +succimer (5mg/k)	-0.238	0.554	-0.068
B. Pinnatum+100mg/kg	0.427	-0.186	-0.362
B. pinnatum+200mg/kg	-0.340	-0.331	0.414
Succimer (5mg/kg)Alonr	-0.509	-0.045	0.006

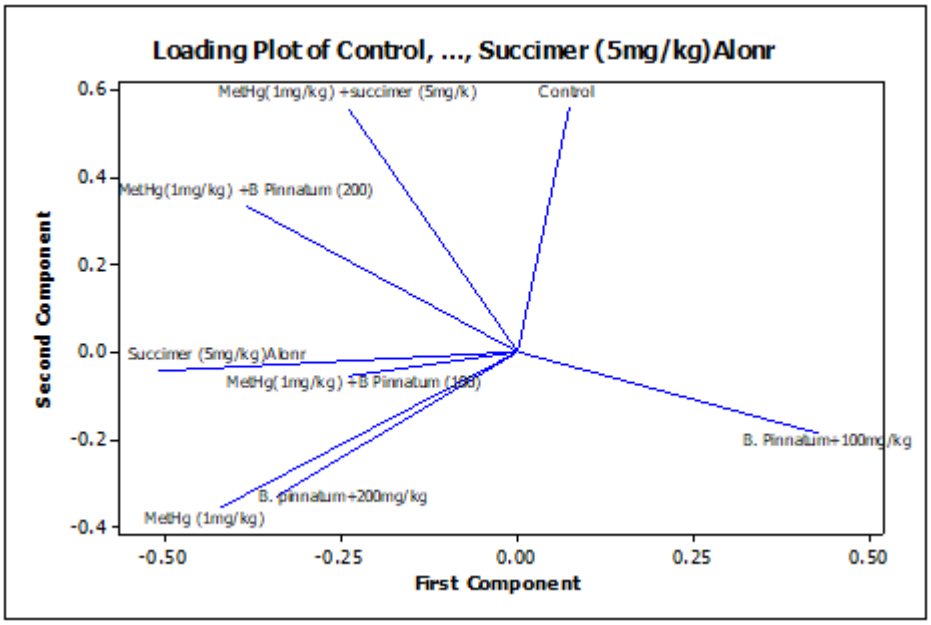

Figure 3: Principal Component Analysis (PCA) of luteinizing hormone (IU/L)

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Step	clusters	level	level	joined	cluster	cluster
1	7	94.3508	0.11298	2 7	2	2
2	6	92.4409	0.15118	4 5	4	2
3	5	89.1448	0.21710	2 8	2	3
4	4	71.7036	0.56593	3 4	3	3
5	3	60.0818	0.79836	2 3	2	6
6	2	43.4221	1.13156	1 2	1	7
7	1	24.7488	1.50502	1 6	1	8

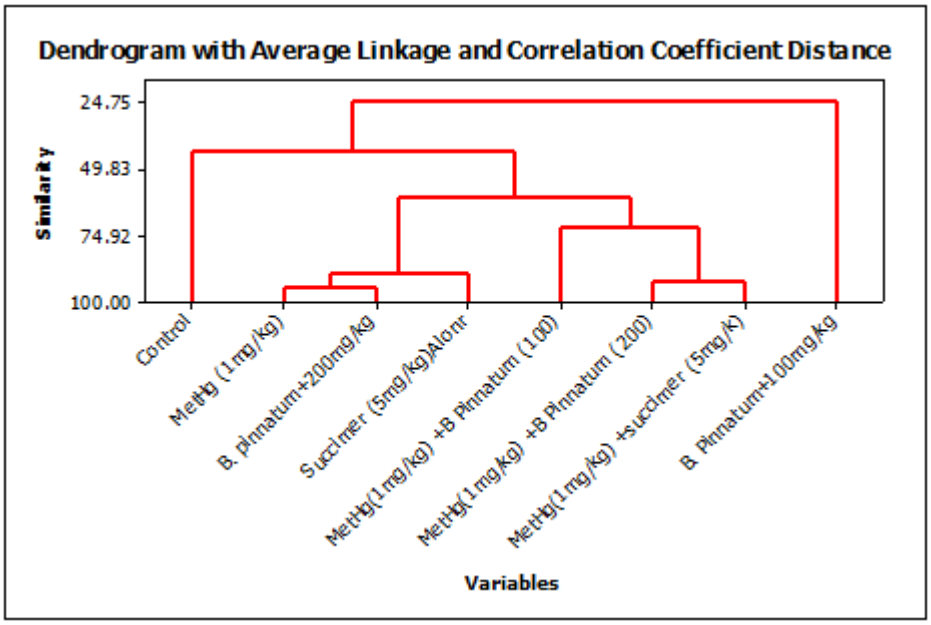

Figure 4: Dendrogram plot for of luteinizing hormone (IU/L)

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