TOXICOLOGICAL EFFECT OF LOCAL/NATURAL INSECTICIDES: SEEDS OF *AZADIRACHTA INDICA*, PEELS OF *CITRUS SINENSIS* AND THEIR COMBINATION ON LIVER ENZYMES

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Abstract

Natural plant products and materials have been used by local people as insecticides to control insects such as mosquito. Among the most notable plants having such insecticidal properties are *Azadirachta indica* (neem) and *Citrus sinensis* (sweet orange). The leaves of these plants have been immensely studied and well known for their medicinal value. Whereas, the seeds of *Azadirachta indica* and peels of *Citrus sinensis* were mostly known for their insecticidal properties and have been found to be hepatotoxic. Therefore, this study is aimed at investigating the toxicological effects of these plant materials on the liver. The treatment involved exposing three groups of white albino rats (Rattus novergicus) to the smoke of dried and grinded seeds of *Azadirachta indica*, peels of *Citrus sinensis* and their combination at different duration of one hour and two hours for each group respectively. The first group (Group A) was not exposed to any of the natural insecticides and was fed normal diet and water, and therefore served as control. After four weeks of the treatment, all the rats were sacrificed and the liver enzymes AST, ALT and ALP were assayed. The serum levels of AST, ALT and ALP for the rats that were treated have been found to significantly increase (at $p<0.05$) when compared to the control. Therefore, the result of this study revealed that these natural insecticides have significant effect on

the serum level of liver enzymes. However, the duration of exposure increases the severity of the effect. This implies that these plant materials could cause liver injury and as such they are toxic to the liver at the duration of exposure studied.

Keywords: Natural Insecticides, Liver Enzymes, *Azadirachata indica*, *Citrus sinensis*, Toxicity, Liver Injury.

Introduction

Peasant farmers have traditionally used a wide variety of natural products with insecticidal properties including minerals, oils, and vegetable extracts from plant and trees (FAO, 2009). Natural insecticides, such as nicotine, pyrethrum and neem extracts are made by plants to offer protection against insects. Nicotine-based insecticides are widely used in the US and Canada, but are excluded in the European Union (Lavoisier, 2014). The purpose of using of all these natural products is to kill, repel, or otherwise interfere with the damaging behavior of insect pests(Murray *et al.*, 2013).

In general, exposure to pesticide is associated with toxicity among nontargets including humans at varying degrees, and if not used in a proper way, organic insecticides can harm people and the environment (Murray *et al.*, 2013).

Azadirachta indica (neem) is a fast growing tree which is native to the Indian subcontinent, where its medical and insecticidal properties are well known and characterized (Sufia and Chatarjee, 1991; Prabhu and Singh, 1993; Mitchell *et al*., 1997). Mean while, it is widely distributed throughout southeast Asia, East and subsahelian Africa, Fiji Mauritius and parts of central America (Pingale, 2010). The active material with insecticidal properties is azadirachtin and is mainly found in the seeds and bark of the tree (Schmutterer, 1990).

According to Dreyer (2009), Margosan–O is the first commercial insecticide that has derived from the seeds of the neem tree and is registered by the environmental protection agency. The repellent is particularly effective against the Anopheles Mosquito which spreads malaria.

Methanolic and Ethanolic extracts of *Azadirachta indica* seeds oil have been found to have severe hypoglycemic properties, but on the other hand they have significantly increased liver enzymes AST, ALT and ALP in diabetic mice which reveal their hepatotoxicity (Muhammad *et al*., 2013). Various neem seed preparations such as aqueous neem seed Kernel extract demonstrated toxicity to oreochromis niloticus (tilapia) and cyprinus carpio (cap) (Biswas *et al*., 2002). Another feeding trial with neem seed meal (2.5%) on chicks indicated mild to severe changes in kidney, liver, spleen,

intestine and heart. Likewise, retardation of spermatogenesis was observed by feeding neem seed cake to rats (Biswas *et al*., 2002).

The *Citrus sinensis* (Sweet Orange), especially oranges, are one of the world's four major fruit crops, along with grapes, bananas and apples. Commercial citrus species are native to south – East Asia and eastern India (Milind and Dev, 2012). Most present day cultivars have been grown for many years (Hartmann *et al.*, 1988).

Volatile constituents and flavonoids of extracts of citrus fruits have been suggested to have properties against fungus and insects (Macias *et al*., 2005). Statistical studies using the randomized complete block design with four replicates showed that volatile extracts of two species of orange peels, *citrus sinensis* (sweet orange) and *citrus aurantifolia* (Lime) had insecticidal activity against mosquito, cockroach and housefly (Ezeonu *et al*., 2001).

The active ingredient, D-limonene, destroys wax coatings of the insect's respiratory system when applied directly by suffocating insects. The citrus fragrance also acts as a repellent(Ezeonu *et al*., 2001). The EPA calls D-limonene a broad-based insecticides. It can be used for aphids, ants, mealy bugs, gnats, silver fish, fleas, mosquito, cockroaches and house flies (Ezeonu *et al*., 2001).

Limonene is moderately toxic by ingestion and its poisoning may affect the kidneys. The oral LD_{50} is 5g/Kg for a rat and 5.6g/Kg for a mouse, local effects include irritation to the eye, skin and respiratory tract. Acute exposure may cause sore throat, coughing', shortness of breath (diptheria, dizziness and nausea). Limonene is also a possible carcinogenic agent (Steward, 1994).

Liver function tests are groups of Clinical Biochemistry laboratory blood assays designed to give information about the state of a patient's liver (Lee, 2009). Four separate liver enzymes are included on most routine laboratory tests. They are aspartate aminotransferases (AST or SGOT) and alanine aminotransferase (ALT or SGPT), which are known together as transaminases; and alkaline phosphatase (ALP) and a gamma-glutamyl transferase (GGT) which are known together as cholestatic liver enzymes (Johnston, 1999; McClatchey and kenneth, 2002). Elevations of these enzymes can indicate the presence of liver disease, but ALT is more liver specific than AST and therefore give an excellent indication for liver injury. However, elevation of ALT is usually accompanied by similar elevation of AST and other liver enzymes (Moss and Henderson, 1999; Palmer, 2004).

This study was conducted within the overall aim of determining the safety of natural insecticides to the public. This is because, In previous years and even presently in some villages, people are in the habit of using some local or natural plant products/parts to control insect pests without being informed about the side effects of such products on the vital organs of

the body (such as liver, heart and kidney). Therefore, it is very essential to determine the effects of these natural insecticides on the users so as to minimize the toxicity.

Materials And Methods

Experimental Animals

Twenty one healthy males and females white albino rats of an inbred Novergicus strain (*Rattus novergicus*) weighing between 120g to 240g were obtained from the Animal Holding Unit of department of Biological Sciences, Faculty of Sciences, Bayero University Kano and Animal Holding Unit of department of Pharmacology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria. The rats were housed in a well ventilated environment (Temperature: 25-30^{o}; Photoperiod: 12 Hours natural light and 12 hours dark), kept in aluminum cages (Dimensions 39.50cm by 25.30cm by 14.80cm) with saw dust at the bottom of the cages and they were fed with Animal Pelletized Feed (Growers Feed) made in "Bukuru"Jos, Plateau State with free access to water.

Experimental Design

The rats were distributed on the basis of their weight into seven groups (A to G) of three rats each, with group C, E and G having the highest weight because they were exposed to the natural insecticides for longer duration. Group A which served as control was given normal food and water. The animals in the other groups (B to G) were exposed to the smoke of the local insecticides in an inhalation chambers (Dimensions 119cm by 61cm by 55cm). Groups B and C were exposed to smoke of neem seeds for one hour and two hours respectively. Likewise, Group D and E were exposed to smoke of orange peels, in which group D was exposed for one hour and group E for two hours. Then, Groups F and G were exposed to smoke of the combination of neem seeds and orange peels for the duration of one hour and two hours respectively.

One of the three rats in group G died on the 23rd day of the treatment, most likely because of the longer duration of exposure. The treatment was carried out daily for period of four weeks.

All the rats were sacrificed on the 29th day by carefully cutting their juguler vein with a very sharp razor. Blood samples were collected into dried and clean centrifuge tubes allowed to clot and centrifuged. The sera were collected into plain serum bottles for laboratory investigations.

Animal Ethics

The animals used in this experiments were treated according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals

prepared by the National Academy Science which was published by National Institute of Health.

The ethic regulations have been followed in accordance with National and Institutional Guide Lines for the protection of animal's well being during experiments(Public Health Service, 1996).

Collection and Preparation of Samples

The fruits of *Azadirachta indica* (neem) were collected from Bayero University, Kano which is within Gwale local government area of Kano state, Nigeria. Whereas the peels (bark) of *Citrus sinensis* (sweet orange) were collected from "Hauren wanki" of Gwale local government of Kano state. Both the fruits and the peels were identified at Botanical garden of department of Biological Sciences, Bayero University, Kano.

The seeds of the *azadirachta indica* were exposed out of the fleshy fruits (depulped). Both the seeds and peels were sun dried (in order to reflect the actual traditional processing procedures) and ground into powdered form using pestle and mortar. The powder was then stored in a well dried and clean container at room temperature.

Enzymes Assay

All the liver enzymes were assayed using a commercially prepared kits, AST and ALT were analyzed using Randox commercially prepared kit, but ALP was analyzed using TECO commercially prepared kit. Standard clinical laboratory procedures were employed while assaying all the liver enzymes.

Results

The effects of exposure of experimental rats to the smoke of natural insecticides powders: seeds of *Azadirachta indica*, peels of *Citrus sinensis* and the combination of the two on the activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP) were presented below (Tables 1 and 2).

Table 1: Serum enzymes activities of AST, ALT and ALP of rats exposed to the smoke of natural insecticides one hour daily for four weeks.

Treatments	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Group B (Neem Seeds). n=3	34.33 ± 2.89^{ab}	14.33 ± 2.52	60.00 ± 14.53^{a}
Group D (Orange peels). n=3	34.33 ± 2.89^{ab}	13.67 ± 3.06	43.34 ± 5.77^{a}
Group F (combination). n=3	25.67 ± 2.31^{a}	12.67 ± 3.79	42.22 ± 10.72
Group A (control). n=3	17.00 ± 1.73	10.67 ± 0.58	28.89 ± 3.85

The results were expressed as: mean ± standard deviation
n = number of experimental animals
"a" indicate significant difference when compared with Group A ($P < 0.05$).
"b" indicate significant difference when compared with Group F ($P < 0.05$).

Table 1 revealed a significant increase in serum AST levels of rats in group B, D and F when compared with Control (Group A), but no significant difference was observed in the case of ALT for all the groups. Whereas, for ALP only group B and D showed a significant increase in serum enzyme activities when compared to control. Only serum AST level was significantly increased when group B and D were compared with group F.

Table 2: Serum enzymes activities of AST, ALT and ALP of rats exposed to the smoke of local insecticides two hours daily for four weeks.

Treatments	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Group C (Neem Seeds). n=3	44.33 ± 16.17^a	16.00 ± 1.73^a	51.11 ± 1.92^a
Group E (Orange peels). n=3	41.00 ± 0.00^a	21.00 ± 2.65^a	46.67 ± 12.02
Group G (combination). n=2	33.50 ± 3.54^a	18.00 ± 1.41^a	35.00 ± 7.07
Group A (control). n=3	17.00 ± 1.73	10.67 ± 0.58	28.89 ± 3.85

The results were expressed as: mean ± standard deviation
n = number of experimental animals
"a" indicate significant difference when compared with Group A ($P < 0.05$).

Table 2 revealed a significant increase in serum levels of both AST and ALT for the rats in group C, E and G when compared with the control rats (Group A). But for ALP, only group C indicated a significant difference when compared with control. However, no any significant difference was observed in all the three liver enzymes, when group C and E were compared with group G.

From the above tables (1 and 2), statistical comparisons between the results were done using student's t-test at 5% level of significance as described by Mukhtar (2003). Significant difference was observed when Group D and Group E were compared at $P < 0.05$ for AST and ALT, whereas for ALP, no significant difference was observed.

Discussion

The result of this study revealed a significant increase at ($P<0.05$) in the serum activities of liver enzymes: Aspartate aminotransferase, Alanine aminotransferase and Alkaline phosphatase upon exposure of experimental rats to the smoke of natural insecticides (seeds of *Azadirachta indica*, peels of *Citrus sinensis* and their combination). However, This could be due to the necrosis or cytolysis of the hepatocytes by various toxic phytochemicals

present in both *Azadirachta indica* and *Citrus sinensis* such as Azadirachtin and Limonene which probably cause leakage of the liver enzymes into the blood. This implies that these local insecticides cause hepatic injury which means that they have toxic effect on the liver of the experimental animals. This finding is consistent with the findings of Ashafa *et al* (2012) who reported the toxic effects of *Azadirachta indica* and Tarkang *et al* (2012) who reported the toxic effects of *Citrus sinensis* on the liver and other vital organs of experimental rats.

Serum AST activity significantly increased in both one hour and two hours exposure of the rats to all the natural insecticides, and this is because other factors besides liver injury could cause increase in AST owing to its less specificity to the liver. Whereas, ALT activities did not show any significant increase for all the local insecticides in one hour exposure, but do so in two hours exposure. This implies that the effect of these natural insecticides on the liver is not manifested in one hour until two hours, because ALT is highly specific to the liver and therefore give an excellent indication of liver injury (Jacobs *et al*., 1994). Likewise, in the case of those rats exposed to the orange peels, increasing the duration of exposure (from one hour to two hours) increases the severity of AST and ALT activities in the serum. Therefore, it is generally implied that the duration of exposure plays a great role in the degree of increment in AST, ALT and ALP activities in the serum of experimental animals, which in turn shows increase in severity of the liver toxicity. Furthermore, the result of this study showed that combination of the two local insecticides does not have a synergistic effect on the activities of AST, ALT and ALP.

Serum total ALP levels provide a useful but nonspecific indication of liver or bone disease with billiary tract obstruction which leads to its diffusion into the blood. The serum ALP levels increased significantly in both one hour and two hours treatments at ($P< 0.05$) and was consistent with the finding of Brown *et al*. (2007).

The measurement of the activities of markers or diagnostic enzymes in the serum plays a significant role in diagnosis of diseases and in the assessment of drugs or plant extract for safety or toxicity risk. The enzymes considered in this study (AST, ALT and ALP) are useful marker enzymes of the liver cells (Schmidt and Schmidt, 1979).

Conclusion

The results of this study have shown that daily exposure of the experimental rats to the smoke of natural insecticides: seeds of *Azadirachta indica,* peels of *Citrus sinensis* and their combination for the duration of one hour and two hours for four weeks have significantly increased the level of serum liver enzymes. Also increasing the duration of exposures from one

hour to two hours, increased the degree of increment of these liver enzymes in the serum, which reflects the increase in the severity of liver damage with time. But combining the two local insecticides together (neem seeds and orange peels) did not have a synergistic effect on the level of serum liver enzymes but rather decreased the effect of individual insecticides. As such, within the limit of experimental error, the pattern displayed by the liver enzymes in this study indicated that these local insecticides may cause liver injury and as such they are toxic to the liver at the specific duration of exposure.

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