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Safety Assessment of Neem (*Azadirachta indica*) Leaf Extracts on BALB/c Mice for Anticancer Therapy

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ABSTRACT

***Azadirachta indica* commonly known as neem, has a wide range of medicinal properties. It is known to have antiallergenic, antidermatic, antifungal, anti-inflammatory and other biological activities. Anticancer properties of neem has also been tested in the recent past. Almost all the parts of the neem tree particularly leaves, bark, seed oil and their purified products were tested for treatment of cancers. However, the safety level of neem leaf extract has not been tested. So the present study is to investigate whether there is any toxic nature of different preparations of neem leaf extract such as ethanolic neem leaf extract (ENLE) and modified neem leaf extract (MNLE) in a mouse model. For this study, separate groups of BALB/c mice were subcutaneously injected with two different doses (250 mg/kg body weight and 500 mg/kg body weight) of ENLE and MNLE, and distilled water as the control in every two days until 21 days. Three mice from each group were sacrificed on 7th day and 14th day after treatments to obtain blood to determine the hepato and renal toxicity and body organs for histopathological tests. To determine the hepato and renal toxicity, serum parameters serum gluatmic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum creatinine were tested. According to the present study, MNLE didn't cause any lethality or produce any remarkable renal and hepato toxicity or histopathological signs. The ENLE elicited significant increase in serum enzyme levels as well as highest damage to the liver tissues. However, high serum enzyme levels recorded from the groups, which are treated with ethanolic leaf extract became normal with the time but the damage of the liver tissues did not recovered. Thus, the present study confirmed that MNLE is safer than the ENLE. Therefore, further improving of modified neem leaf extract upto the required standards, which can be recommended to use as a medicine in the future.**

KEYWORDS: *Azadirachta indica*, Modified neem leaf extract, Toxicity

INTRODUCTION

Cancers figure as one of the foremost reasons of morbidity and mortality worldwide. It claims more than six million people's lives a year (Akhir *et al.*, 2011). Cancer is a generic term for a large group of diseases that can affect any part of the body. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. For the management of cancers there are so many treatments such as, surgery, chemotherapy and radiation therapy (World Health Oraganization report, 2015). But the new trend is use of herbal medicines in order to treat cancers, as most of the chemical treatments cause to large number of side effects.

Herbal medicines involve plant derived agents. Some medicinal plants have therapeutic potential due to the presence of natural antioxidants. Superior part of their antioxidant action is due to bioactive compounds, which are present as secondary metabolites (Nema *et al.*, 2012).

Among all medicinal plants, neem (*Azadirachta indica*) has an important place due to its large number of medicinal properties. It is a fast growing ever green tree in the family *Meliaceae* and it has been attracted in the medical field in recent years. The medicinal properties of the plant neem was studied by several workers. Different parts of neem tree is known to have antiallergenic, antidermatic, antifungal, anti-inflammatory, antiscabic, and other biological activities. All these biological activities are due to several biologically active compounds such as, azadirachtin, meliacin, gedunin, salanin, nimbin, valassin *etc.* (Pankaj *et al.*, 2011).

During last decade, neem was investigated in detail to identify its anticancer properties. Almost all the parts of the neem tree, particularly leaves, bark, seed oil and their purified products are widely tested for treatment of cancers.

Effect of ethanolic neem leaf extract on cancers was reported as, it can induce the apoptosis in 4T 1 breast cancer cells in a mouse model (Othman *et al.*, 2011). In another study reported that, the ethanolic leaf extract of neem fight against buccal carcinogenesis, skin carcinogenesis, prostate cancer, mammary carcinogenesis, gastric carcinogenesis, also B16 melanoma (Arisanty, 2013).

Although there are so many information about the numerous medicinal properties of neem, but the possible toxicity that neem may cause to the consumers has not been studied much. Plants or drugs must be ensured to be safe before they could be used as medicines. A key stage in ensuring the safety of drugs is to conduct the safety assessment tests in appropriate animal models.

The objective of the present study is to determine the level of toxicity of different preparations of neem leaf extract before it can be used as an anticancer drug.

MATERIALS AND METHODS

Experimental Site

The study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka with the collaboration at Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, and University of Peradeniya and at Department of Histopathoogy, Medical Research Institute, and Colombo 8, during the period of December 2015 to May 2016.

Experimental Animals

Healthy, adult female BALB/c mice (25-30 g) were purchased from the Medical Research Institute, Colombo 8, Sri Lanka. Total of 25 mice were divided in to five groups and housed in the Faculty of Medicine, University of Peradeniya.

Sample Collection

Healthy immature neem leaves were collected from Kuliyapitiya area for the preparation of ethanolic and modified neem leaf extracts.

Blood samples were collected into heparin contained containers from randomly selected three mice from each group by heart puncture. Serum was separated by centrifugation at 2,500 rpm, 10 min and stored in -20 °C freezer.

Body organs of mice were collected into 10% formalin contained containers from same three randomly selected mice, which were used to collect blood samples. Samples were stored at room temperature until used for the histopathological examination.

Preparation of Neem Leaf Extracts

Ethanolic neem leaf extract (ENLE) was prepared using previously described method with few modifications (Othman *et al.*, 2011). Dried *Azadirachta indica* leaf powder was mixed with 200 mL of 80% ethanol and kept overnight at room temperature. Then the mixture was filtered into a beaker and another 200 mL of 80% ethanol was added to the remaining residue. Again it was kept at room temperature overnight. These steps were repeated for another three consecutive days.

Modified neem leaf extract (MNLE) was prepared using previously described method with slight modifications (Warusawitharana and Vivehananthan, 2010). Hundred grams of dried neem leaf powder was mixed with 200 mL of distilled water and mixture was filtered through a muslin cloth. Then the extract was modified by adjusting the pH.

Then both modified and ethanolic leaf extracts were evaporated separately using a rotary evaporator at 55 °C. After that the extracts were dried using a freeze drying system at -80 °C for 24 h and then 48 h in the oven at 40 °C and stored at 4 °C.

Differential Cell Counts of Mice

Two different concentrations [(C_1) 250 mg/kg, and (C_2) 500 mg/kg] of ENLE (T_1) and MNLE (T_2) were subcutaneously injected to four groups of mice in every two days until 21 days. Distilled water was used as the control (CONT). Blood was taken from the tail of randomly selected three mice from each group at weekly interval upto one month period and blood smears were prepared. Then the different white blood cells count were taken using a light microscope after stained with leishman stain.

Toxicity Assay of Neem

One milliliter of blood was collected from three randomly selected mice from each group on 7th day and 14th day after treatments by heart puncture. Serum parameters (Serum Gluatmic Oxaloacetic Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), and Serum Creatinine) were determined using Human kits (Human GmbH, Max-Planck Ring 21, 65205 Wiesbaden, Germany) and then analyzed by using biochemistry analyzer (Chem-7, Erba Mannheim, Germany) as per manufacturer's instructions.

Histological Examination of Liver and Kidney

Liver and kidney of mice were examined on the 7th and 14th day after treatments to

determine whether there is any toxic effect of the neem leaf extracts.

Animals were sacrificed to obtain the liver and the kidney. Then the organs were immediately fixed in 10% formalin, dehydrated with alcohol and embedded in paraffin. After that tissues were cut into 3-4 μm thick sections by microtome, and stained with haematoxylin and eosin dyes for microscopic observation. The microscopic features of the organs of mice in treated groups were compared with the control group mice by using light microscope.

Statistical Analysis

Statistical software, GraphPad Prism 5 Demo was used to analyze the significance difference between the control and the treatment groups.

RESULTS AND DISCUSSION

Differential Cell Counts of Mice

White Blood Cells are an important part of the immune system. They help to fight off diseases. Among all the WBCs, lymphocytes and the neutrophils temporarily rise in response to a number of conditions or disorders, including infections, injuries, inflammatory disorders, and certain drugs. However, the normal ranges of these different cells are in range of lymphocytes (70-75%), neutrophils (20-25%), monocytes (3-4%), and eosinophils (0-2%), basophils (0-2%).

Differential cell counts with the treatment of both types of extracts were normal throughout the experiment period, indicating that all the mice were in good health condition throughout the experimental period (data not shown). It was a good sign to continue the rest of the experiment.

Determination of Toxicity Level by Serum Parameters

Liver is the organ, which breaks down substances that we take by mouth, including medications, herbs and supplements. This process usually takes place efficiently without causing any harm to the body. Whenever new medications are developed, they are extensively tested prior to being approved for general use.

Not only the liver, have kidneys also play an important role by filtering out the toxins from the body. As well as kidneys maintain the blood creatinine in a normal range. Which is a waste product produced in the muscles. Elevated creatinine level signifies impaired kidney function, kidney disease or damage to the kidney cells.

Therefore, testing of the serum enzyme levels of both liver and kidney is important to detect the toxic nature after treating with two different doses (250 mg/kg and 500 mg/kg) of different preparations of neem leaf extracts.

Serum Gluatmic Oxaloacetic Transaminase (SGOT)

The level of SGOT of the four treatment groups were not significantly different ($P>0.05$) compare to the control group before start the treatments (Figure 1). It was in the normal range same as the reference range (54-298 U/L) reported by Research Animal Resources, University of Minnesota.

However, when it comes to the 7th day after treatments there was a significant difference ($P<0.05$) in all four treatment groups compare to the control group. The groups treated with the both doses of MNLE showed significantly lower values (49.275 U/L for 250 mg/kg and 13.55 U/L for 500 mg/kg) than the values obtained for the ENLE treated groups. The highest value (416.35 U/L) was recorded for the higher dose of ethanolic extract (T_1C_2) treated group. At the 14th day after treatments only the lower dose of modified extract (T_2C_1) treated group reached to the normal range (59.66 U/L).

According to the results obtained throughout the experiment, the toxic effect caused by the two different extracts (ENLE and MNLE) were significantly different. These observations may be due to the quality of the modified neem leaf extract, as it has been previously proved (Warusawitharana and Vivehananthan, 2010). Therefore, safer use of MNLE is confirmed through this study.

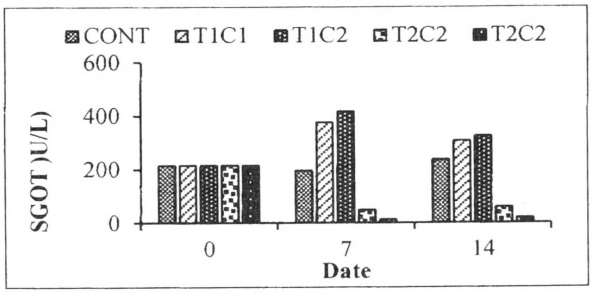

Figure 1. Mean SGOT levels of adult female BALB/c mice before start the treatments, 7th day and 14th day after treatments. CONT- control, *T_1C_1- ENLE low dose, T_1C_2- ENLE high dose, T_2C_1- MNLE low dose, T_2C_2- MNLE high dose*

Serum Glutamate Pyruvate Transaminase (SGPT)

The level of SGPT detected in the four treatment groups were not significantly different ($P>0.05$) compare to the control group before start the treatments and they were in the normal range (Figure 2) as same as the reference range (17-77 U/L) reported by Research Animal Resources, University of Minnesota. When it comes to the 7th day after

treatments there was a significant difference ($P<0.05$) in four treatment groups compare to the control group. But the values obtained for the two different extracts were not significantly different from each other's except in high dose of modified extract treated group (T_2C_2). However, at the 14th day after treatments, the lower dose of the modified extract treated group (T_2C_1) reached to the normal range (76.76 U/L) indicating the lower damage to the liver from the MNLE compare to the ENLE. That may be due to the improved quality of the modified leaf extract without any possible contaminants which was not removed in the ENLE, in the present study.

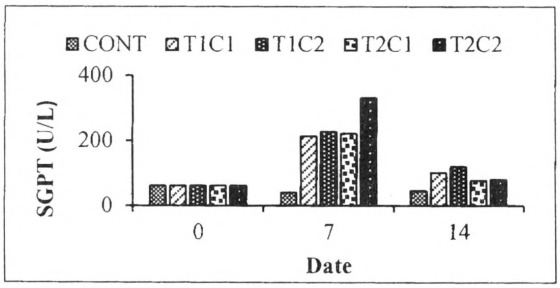

Figure 2. Mean SGPT levels of adult female BALB/c mice before start the treatments, 7th day and 14th day after treatments. CONT- control, *T_1C_1- ENLE low dose, T_1C_2- ENLE high dose, T_2C_1- MNLE low dose, T_2C_2- MNLE high dose*

Serum Creatinine

In the present study, the level of serum creatinine in all the mice were in normal range (0.2- 0.9 mg/dL) before start the treatments. But it was slightly elevated in three treatment groups except in high dose (500 mg/kg) of modified extract (T_2C_2) treated group (0.795 mg/dL) when it comes to the 7th day after treatments (Figure 3). The highest value (1.02 mg/dL) was recorded with high dose of ethanolic extract (T_1C_2) treated group and it was not significantly different ($P>0.05$) from the control group mice. At the 14th day after treatments all the values in four treatment groups became normal indicating that both types of neem leaf extracts do not cause for any damage to the kidney.

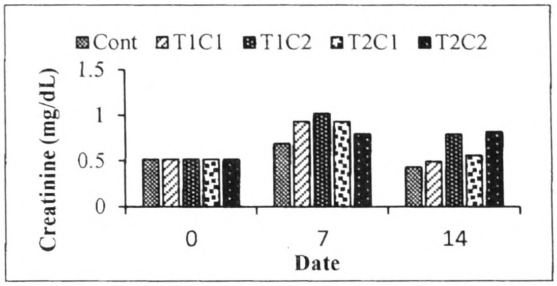

Figure 3. Mean S. Creatinine levels of adult female BALB/c mice before start the treatments, 7th day and 14th day after treatments. CONT- control, *T_1C_1- ENLE low dose, T_1C_2- ENLE high dose, T_2C_1- MNLE low dose, T_2C_2- MNLE high dose*

Histopathological Effect of Neem on Liver and Kidney Tissues

Further, toxic nature of neem leaf extracts were investigated by histopathological analysis indicated in Table 1. Only the liver tissues showed slight damage compare to the control group. At the 7th day after the treatments, all four treatment groups showed all most same damaging pattern in liver tissues with mild to moderate sinusoidal dilatation and edema of hepatocytes (Table 1). When it comes to the 14th day after treatments only the MNLE treated groups showed recovering signs of damaged liver cells with mild sinusoidal dilatation and absence of edema of hepatocytes. But the ENLE treated groups didn't express recovering sings and they showed development of moderate focal and diffuse mixed inflammatory cells infiltration. This difference between MNLE treated groups and the ENLE treated groups may be due to the high purity of the MNLE.

Histopathological examination of the kidneys in ENLE and MNLE administrated groups showed no differences from the control group (data not shown), indicating that completely no damage to the kidney by injecting two different neem leaf extracts at these concentration levels by confirming that there is no any renal toxicity due to the neem leaf extracts.

Table 1. Histological results of Liver of adult female BALB/c mice 7th day and 14th day after the treatments

	7th day after treatments			14th day after treatments		
	Sinusoidal dilatation	Edema of hepatocytes	Inflammatory cells infiltration	Sinusoidal dilatation	Edema of hepatocytes	Inflammatory cells infiltration
CONTROL	Absent	Absent	Absent	Absent	Absent	Absent
T_1C_1	Mild	Mild	Absent	Mild	Mild	Moderate
T_1C_2	Moderate	Mild	Absent	Moderate	Mild	Moderate
T_2C_1	Moderate	Mild	Absent	Mild	Absent	Absent
T_2C_2	Moderate	Mild	Absent	Mild	Mild	Absent

T_1C_1- ENLE low dose, T_1C_2- ENLE high dose, T_2C_1- MNLE low dose, T_2C_2- MNLE high dose

CONCLUSIONS

Modified neem leaf extract can be recommended to be safe in lower dose (250 mg/kg) as it didn't cause any lethality or produce any renal and hepato toxicity or remarkable histopathological signs. But ethonolic neem leaf extract caused mild hepto toxicity. Therefore, modified neem leaf extract can be suggested, to be used in drug development in future, after improving the quality of neem leaf extract.

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