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Suppression of Ki-67 immunomarker and prostate specific antigen by saponin fraction of *Vitex doniana* leaf in nitrosobis (2-Oxopropyl) amine-induced prostate toxicity

Ifeanacho Ezeteonu Abireh[1], Godson Emeka Anyanwu[2]
[1] Enugu State University of Science and Technology, Enugu, Nigeria
[2] University of Nigeria, Enugu, Nigeria

Abstract
Aim: This study investigated the suppressive effect of saponin from *Vitex doniana* leaf on Ki-67 immunomarker and prostate specific antigen in prostate toxicity induced with Nitrosobis (2-oxopropyl) amine.
Material and Method: The male Wistar rats used for this experiment were grouped into 5 groups of 4 rats each. The experiment lasted for 28 days. From day 1-14, group 1 received subcutaneous normal saline 0.5ml daily, while groups 2, 3, 4, and 5 received Subutaneous Nitrosobis (2-oxopropyl) amine 5mg/kg daily. From day 15-28, group 1 received oral normal saline 0.5ml daily, while groups 3, 4, and 5 received oral saponin at 250mg/kg, 500mg/kg, and 750mg/kg daily, respectively. Group 2 did not receive nitrosobis (2-oxopropyl) amine nor saponin from day 15-28. All the rats had free access to commercial rat chow throughout the experiment.
Results: Significant increase ($p<.05$) in prostate Ki 67 immunomarker expression and serum prostate specific antigen level were observed in group 2 (Nitrsobis amine only) when compared with group 1 (normal saline). There was decrease in the level of expression of Ki-67 immunomarker in group 3 (Nitrosobis amine plus Saponin 250mg/kg), and group 4 (Nitrosobis amine plus Saponin 500mg/kg when compared with group 2. However, this decrease was not statistically significant ($p>.05$). There was statistical significant decrease ($p<.05$) in Ki-67 expression in group 5 (Nitrosobis amine plus Saponin 750mg/kg) when Compared to group 2. Significant decrease in the level of serum prostate specific antigen was observed in groups 4, and 5, when compared to group 2. There was no significant decrease in prostate specific antigen level in group 3 when compared to group 2.
Conclusion: Saponin fraction of *Vitex doniana* significantly reduced the prostate Ki-67 immunomarker expression and serum prostate specific antigen concentration in the dose studied. Thus saponin may be an agent that could be employed to reduce disease arising from prostate toxicities, such as prostate cancer.

Keywords: Ki-67, Prostate specific antigen, saponin, *Vitex doniana*, nitrosobis amine

Introduction

Ki-67 is an important immunomarker used in pathology and clinical setting to ascertain cellular proliferation, and help grade different cancers [1]. Ki-67 is identified using immunohistochemistry assay of the tissue being examined following extraction from the animal [2]. Prostate specific antigen, a protease that is produced by the prostate, is a marker used for assessment of different toxicities of the prostate [3]. It is elevated in prostatitis, benign prostatic hyperplasia, and prostate cancer [4]. Nitrosobis (2-oxopropyl) amine belongs to Nitrosamines that cause DNA methylation and cause cancer [5, 6]. Cancer affecting the prostate is the most common diagnosed malignancy in men [7]. The risk factors for prostate cancer include race, genetics, and obesity [8]. Other risk factors include diet, cigarette smoking [9]. Several options of treatment of the diseases of the prostate are available, but these available options have not been able to reduce the growing incidence and mortality associated with the disease, especially prostate cancer, as it still accounts for several deaths worldwide [7]. So seeking a lasting option of treatment have become necessary in order to mitigate this rising trend in mortality.
Various parts of different plants have served as sources of several medications that are currently in use for treatment of various diseases [10,11]. Some studies have shown that *Vitex doniana* leaf have potential in the treatment of cancers including breast cancer [12], and prostate cancer [13]. The antineoplastic effects of *Vitex doniana* have been attributed to several of its phytochemical contents, which include saponins, tannins, anthraquinones, terpenoids, and flavonoids [14, 15].
Several cancers such as colon cancers, pancreatic cancers, and breast cancers have responded favorably to treatments with saponin [15, 16]. However, there is no study on the use of saponin from Vitex doniana leaf in the treatment of prostate cancer. This study investigated the suppression of cellular expression of Ki-67 immunomarker and serum concentration of prostate specific antigen by saponin fraction of *Vitex doniana* leaf in nitrosobis (2-oxopropyl) amine-induced prostate toxicity in Wistar rat.

Materials and Method

Preparation of extract

The stalks of the leaves of Vitex doniana were removed, and the leaves washed with distilled water and allowed to dry at room temperature. After drying, the leaves were made into powder form using mortar and pestle. Then sieved off of ungrounded fibers. At each time, 1000g of the powdered leaves was extracted. After extraction, the aqueous extract was filtered using Whatman No. 2 filter paper. Rotary

evaporastor set at 40°C was used to concentrate the extract [17].

Isolation of saponin

Saponin was extracted from the extract using the method described by Majinda (2012). The extract was concentrated under reduced pressure and partitioned successively using n-hexane, ethyl acetate, and n-Butanol. The n-Butanol soluble fraction and the aqueous part afford the major saponin triterpene fraction. The crude extracts were applied separately to columns of Diaion HP-20 which were then washed with Water-Methanol in various ratios (0, 50, 85, and 100) and finally with acetone. The fractions found to have the same pattern were mixed together and separated further by silica gel column chromatography with ethyl acetate-Methanol-water (40:10:1 v / v / v). Then the saponin compounds was separated by HPLC on Octadecylsilyl column using Methanol-water as eluent [18].

Procurement of materials

The Nitrosobis (2-oxopropyl) amine was procured from Sigma-Aldrich, USA. The Wistar rats were procured from the Animal House of Department of Anatomy, Univeristy of Nigeria.

Animal Handling and grouping

Twenty (20) male Wistar rats with average weight of 170g were used for the experiment. The rats were kept in netted iron cages where they were allowed 12-hour light and dark cycles, with temperature of 25°C and humidity of 60-70% throughout the experiment period, according to the protocol of the Committee for the purpose of control and supervision of experiments on Animals. The rats were grouped into five (5) with 4 rats each. Each group of 4 rats was placed in separate clean iron cages, and allowed two (2) weeks for acclimatization before commencement of administration of agents. During this time, they had free access to commercial rat chow and water. The experiment lasted for 28 days.

Administration of agents

From day 1-14, group 1 received subcutaneous normal saline 0.5ml daily, while groups 2, 3, 4, and 5 received Subcutaneous Nitrosobis (2-oxopropyl) amine 5mg/kg daily [6]. From day 15-28, group 1 received oral normal saline 0.5ml daily, while groups 3, 4, and 5 received oral saponin at 250mg/kg, 500mg/kg, and 750mg/kg daily, respectively. Group 2 did not receive nitrosobis (2-oxopropyl) amine nor saponin from day 15-28, as shown in table 1. All the rats had free access to commercial rat chow throughout the experiment.

Table 1: Showing administration of agents

Groups	1-2 weeks	2-4 weeks
1	Normal saline 0.5ml	Normal saline 0.5ml
2	Nitrosobis amine 5 mg/kg	
3	Nitrosobis amine 5 mg/kg	Saponin 250mg/kg
4	Nitrosobis amine 5 mg/kg	Saponin 500 mg/kg
5	Nitrosobis amine 5 mg/kg	Saponin 750 mg/kg

Animal sacrifice and sample collection

The rats were anaesthetized at the end of the experiment, day 29, using intraperitoneal thiopentone 50mg/kg. Blood samples were then collected from the retro-orbital vein of the rats [19], for analysis of serum prostate specific antigen. They were then sacrificed and the prostate gland excised and fixed immediately in formal saline before immunohistochemical analysis for Ki-67 expression.

Sample Analysis

The immunoenzymometric assay method was used for analysis of blood sample for prostate specific antigen. The histological slides of the prostate gland specimens were prepared using the standard histological techniques with immunohistochemical stain for Ki-67. The slides were analyzed using a light microscope, and computer assisted stereology was used for quantitative analysis of the cells.

Statistical Analysis

This was done using Statistical Package for Social Sciences (SPSS) version 23. p-value of .05 or less was considered significant.

Results

Prostate specific antigen

The was significant increase in the serum level of prostate specific antigen in group 2 that was treated with nitrosobis (2-oxopropyl) amine, when compared to group 1, which received normal saline ($p<.05$). Group 3, which received additional oral saponin at 250mg/kg showed a decrease in the serum level of prostate specific antigen, when compared to group 2, treated with nitrosobis (2-oxopropyl) amine alone. However, this observed decrease was not statistically significant when compared with group 2. The serum level of prostate specific antigen in groups 4, and 5 that received additional oral saponin 500mg/kg, and 750mg/kg, respectively, showed statistically significant decrease, when compared to the values in group 2 (treated with nitrosobis 2-oxopropyl) amine alone, as shown below (table 2). These observed significant decrease were dose dependent, with the group treated with 750mg/kg of saponin (group 5) observed to have the highest effect ($p=.03$).

Immunohistochemical analysis of Ki-67 expression

The Ki-67 immunohistochemical assay photomicrograph is as shown in figure 1. The slides were subjected to computer assisted stereology, and the quantitative value of the tissue Ki-67 expression were obtained, and shown in table 2. There was a statistically significant increase in the tissue expression of Ki-67 in the group treated with nitrosobis (2-oxopropyl) amine alone, group 2, when compared with group 1, which received normal saline ($p<.05$). Group 3, which received additional oral saponin at a dose of 250mg/kg and group 4, which received additional oral saponin at a dose of 500mg/kg showed a decrease in the tissue expression of Ki-67 when compared to group 2, that was given nitrosbis (2-oxopropyl) amine. However, these observed decrease in groups 3, and 4, were not statistically significant ($p=.06$). Group 5 that was treated with additional oral saponin at a dose of 750mg/kg showed a significant decrease in expression of tissue Ki-67 when compared to group 2 (treated with nitrosobis (2-oxopropyl) amine alone ($p<.05$).

Table 2: Showing the values of prostate specific antigen and quantitative representation of Ki-67 expression

Assay	1	2	3	4	5
PSA	3.71±0.24	9.67±0.23	7.40±0.18	4.91±0.25	4.38±0.65
Ki-67	5.00±0.41	20.50±0.96	19.50±0.65	18.25±0.48	15.00±0.41

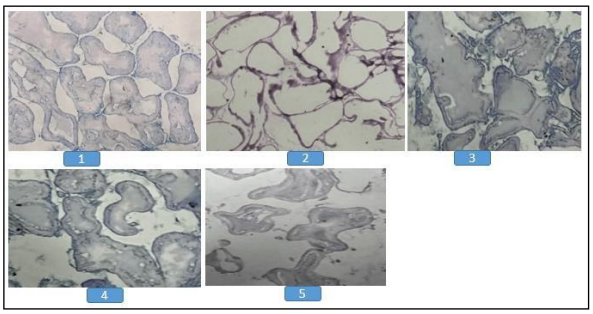

Fig 1: expression of Ki-67 in prostate gland of Wistar rat (x100) of groups 1-5.

Discussion

This study observed that saponin fractionate of *Vitex doniana* leaf suppressed the prostate cellular expression of Ki-67 following initial increase caused by administration of nitrosobis (2-oxopropyl) amine. Also an increase in serum prostate specific antigen caused by nitrisobis (2-oxopropyl) amine was suppressed by the saponin. The reduction in prostate cellular expression of Ki-67 and prostate specific antigen could signify a reduction in burden of prostate toxicity and cancer in the experimental animals [20, 21]. These effects of saponin from *Vitex doniana* leaf observed may be due to its cytotoxic, anti-proliferation, and anti-angiogenesis effect of on cancer cells [22, 23]. Currently, there is no similar study available, but the findings of this study could be correlated with that of Wei et al (2014) who studied the effect of saponin from *Tribulus terrestris* on growth and angiogenesis of human prostate cancer, and found that saponin suppresses the growth of prostate cancer, and also has an anti-angiogenesis effect on the prostate cancer cells [24]. Chen et al., also observed that saponin applied to human prostate cancer PC-3 cells caused an inhibition in migration and invasion of the cancer cells [25]. In another study it was found that ginseng saponin has an inhibitory effect to the growth of human prostate cancer LNCaP, as the prostate specific antigen and other biomarkers such as androgen receptors and proliferating cell nuclear antigen were suppressed following incubation of LNCaP with ginsenoside Rg3 after forty-eight (48) hours [26]. The reduction in expression of Ki-67 by saponin, as observed in this study could also be an indication that saponin from *Vitex doniana* has an inhibitory effect on prostate toxicity and cancer, since increase expression of Ki-67 in prostate cancer is associated with increased gleason score and poor prognosis [27].

Conclusion

The findings of this study suggest that saponin fraction of *Vitex doniana* leaf has an inhibitory effect on prostate toxicity and cell proliferation in experimental animals. However, its exact mechanism of action has not been elucidated. Purification of this saponin may be of great importance in aiding tolerability in humans, with subsequent possibility of its application for the purpose of treatment of human prostate toxicity, such as prostate cancer.

Competing Interest

Authors have declared that no competing interests exist.

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