JOPAT Vol 23(2), 1582- 1597, July – December, 2024 Edition.
ISSN2636 – 5448 https://dx.doi.org/10.4314/jopat.v23i2.15

Ethanol Root Extract of *Uvaria chamae* Down-Regulates Ki-67 in Cadmium Chloride-Induced Prostate Pre-malignancy in Wistar Rats

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Abstract

Prostate malignancy is one of the most diagnosed malignancies in men worldwide and a major health problem to men in both developed and developing world. Currently, several medicinal plants have been reported to possess anti-malignant property, hence we decided to investigate this property in *Uvaria chamae (UC)* on Ki-67 which is a prognostic factor in prostate malignancy. Emphasis was also placed on oxidative stress markers. A total of sixty (60) Wistar rats were used for this investigation and were randomly divided into ten (10) groups of six (6) animals each. Group 1 served as control and received distilled water alone, group 2-6 were induced with pre-malignancy using 3mg/kg of cadmium chloride ($Cdcl_2$) for twenty-eight (28) days. Group 3-6 were subsequently treated with 150mg/kg of casodex, 2500mg/kg, 1500mg/kg and 1000mg/kg of *UC* root extract respectively, after the initial induction of pre-malignancy. Group 7-10 received 150mg/kg of casodex, 2500mg/kg, 1500mg/kg and 1000mg/kg *Uvaria chamae* root extract alone, respectively. After sacrifice, prostate tissues were removed for immunohistochemical assay of Ki-67 and oxidative stress studies. Results obtained from this study showed ki-67 up-regulation in prostate tissues induced with pre-malignancy, similarly a significant down-regulation of Ki-67 marker was observed in prostate tissues of *Uvaria chamae* treated groups after pre-malignancy. Ki-67 marker remained low in *Uvaria chamae* alone treated group. Furthermore, ethanol root extract of *U. chamae* down-regulated tissue level of MDA but up-regulated tissue levels of SOD, CAT and GPx in $Cdcl_2$-induced prostate pre-malignancy. The results established that, *U. chamae* root exhibit strong anti-cancer property in prostate pre-malignancy.

Keywords: *Uvaria chamae*, Ki-67, cadmium chloride, Prostate, Pre-malignancy

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INTRODUCTION

All pre-malignant conditions happen due to the abnormalities in the genetic material of the transformed cells and these abnormalities could be as a result of the impact of carcinogens such as chemicals, tobacco, smoke, radiation or even infectious agents like viruses [1]. About 70% of all deaths associated with prostate cancer occur in developing countries, despite the higher incidence rate in developed nations [1]. This increase in mortality due to prostate cancer may be linked to the radical changes existing in the society due to lifestyle innovations and industrialization [2]. Prostate cancer has a high cure rate and can be effectively treated if it is arrested early. Although prostate cancer is relatively uncommon compared with other forms of cancer, the incidence has been on the increase in recent years in developing countries and has become one of the leading malignant diseases among males between the ages of 65 and above and is one of the major causes of death in Africa, thereby constituting a serious health concern [3-4].

Understanding the risk factors of prostate cancer can help men make the right decisions about their health and potentially screen more often than those without a history of another cancer. It is important that scientists and clinicians establish a practical recommendation for men who have had prostate cancer and who may be at a higher risk of other forms of cancer. In addition, even with the knowledge of the presence of prostate cancer and treatment options particularly within the urban dwellers, the case may not be same within the rural dwellers that may not have access to hospitals, caregivers and drugs but may be fortunate to be surrounded with medicinal plants like *Uvaria chamae*. This is a medicinal plant commonly known as "mmimi ohia" among the Igbos, "kanaika ikot" among the Ibibios, "akisan" among the Yorubas and "ogholo" among the Esan people of Edo state [5]. It is native to Tropical West and Central Africa where it grows in wet and dry forests as well as coastal shrub lands. *Uvaria chamae* has been reported to possess several properties such as anti-inflammatory [5], and anti-oxidant [6]. The Plant has also been reported to cause rejuvenation of seminal vesicle histo-structure in Wistar rats [7]. The problem lies in searching and improvising traditional methods of treatment of cancer apart from chemotherapy, radiotherapy, surgery and hormonal therapy. Radiotherapy for example, used in the treatment of malignant cells have been found to have shortcomings, it randomly kills clusters of cells not taking into consideration whether the cells are cancerous or not and patients undergoing such chemotherapeutic treatment tends to experience painful urination, urethral stricture, rectal bleeding and leaking, lymphodema and erectile dysfunction. Unfortunately, these symptoms may impact negatively on the patient's life and that of the patient's partners [8]. Moreso, other methods such as surgery which is invasive may pose a serious risk including severe pains, post-surgical bleeding and infection. Patients who undergo radical prostatectomy experience post-operative complications which may include urinary incontinence, retrograde ejaculation and erectile dysfunction. This work hopes to unravel the potentials of *Uvaria chamae* in ameliorating carcinogenic potentials and tendencies in the prostate of Wistar rats by examining the down-regulation of a very important pre-malignant marker called Ki-67 as well as evaluating oxidative stress markers. Ki-67 is a promising molecular marker in the diagnosis of pre-malignant and malignant cases and its expression is firmly associated with tumour cell proliferation and growth [9]. This marker is broadly used in pathological investigation as a marker of proliferation.

MATERIALS AND METHODS

Ethical Clearance

Ethical clearance was submitted to the Ethical Committee of School of Basic Medical Sciences, University of Benin, Benin City for approval. Subsequently, approval number CMS/REC/2022/275 was assigned.

Acquisition of Cadmium Chloride, Plant Collection and Identification

Cadmium chloride with batch no. 325755 manufactured by Division Chemical Industry, Miland was purchased from Pyrex chemical shop in Ariaria market, Aba, Nigeria. *Uvaria chamae* root was sourced from a bush in Ikot Efre Itak, a local community in Ikono Local Government Area of Akwa Ibom State. The plant was taken to the Department of Plant Biology and Biotechnology, University of Benin for identification and authentication by a Plant Biologist. Herbarium number UBH-U353 was assigned to the plant.

Plant Extract

About 500 g of the roots of *Uvaria chamae* were air-dried for seven (7) days, pulverized with the use of electric blender and macerated in 70 % ethanol for 3 days. The solution was filtered and taken to water bath to dry at 45 ^{0}C. Obtained dry matter was sieved and preserved in the refrigerator with the use of an air-tight container.

Experimental Animals

Sixty (60) adult male Wistar rats weighing between 200-210g of Wistar species were purchased and bred at the animal house of the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin City. The animals were housed under proper and standard condition, put in clean cages and maintained at room temperature. The animals were given water and standard feed at will. The animals were handled properly according to guidelines for the care and use of animals. The animals were randomly divided into ten (10) different groups, each group was made to contain 6 animals and kept in plastic cages with sawdust used as beddings. Experimental set up for each group is shown below:

Group 1: animals in this group were treated as control. They were provided with only drinking water and feed as and when due.

Group 2: animals in this group were intraperitoneally injected with 3mg/kg of cadmium chloride, once weekly for 28 days.

Group 3: animals in this group were intraperitoneally injected with 3 mg/kg of cadmium chloride, once weekly for 28 days followed by daily/oral administration of 150 mg/kg of casodex for 28 days.

Group 4: animals in this group were intraperitoneally injected with 3mg/kg of cadmium chloride intraperitoneally, once weekly for 28 days followed by daily/oral administration of 2500 mg/kg of *Uvaria chamae* root extract for 28 days.

Group 5: animals in this group were intraperitoneally injected with 3mg/kg of cadmium chloride intraperitoneally, once weekly for 28 days followed by daily/oral administration of 1500mg/kg of *Uvaria chamae* root extract for 28 days.

Group 6: animals in this group were intraperitoneally injected with 3 mg/kg of cadmium chloride intraperitoneally, once weekly for 28 days followed by daily/oral administration of 1000 mg/kg of *Uvaria chamae* root extract for 28 days.

Group 7: animals in this group were treated orally with 150 mg/kg of casodex alone daily for 28 days orally.

Group 8: animals in this group were treated orally with 2500 mg/kg of *Uvaria chamae* root extract alone, daily for 28 days.

Group 9: animals in this group were treated orally with 1500 mg/kg of *Uvaria chamae* root extract alone, daily for 28 days.

Group 10: animals in this group were treated orally with 1000 mg/kg of *Uvaria chamae* root extract alone, daily for 28 days.

Immunohistochemistry and Oxidative Stress Studies

At the conclusion of administration regimen, the rats were subjected to chloroform inhalation and sacrificed immediately. A vertical incision from the abdomen to the pelvis was employed to access the prostate gland. The prostate tissues were fixed in 10% buffered formalin solution for immunohistochemical assay of Ki-67. More so, prostate tissues were obtained for oxidative stress studies (MDA, SOD, CAT and GPx).

Microscopy and Cell Counting

Photomicrographs were obtained at x100 magnification after viewing with a light microscope (Primo star 415500-0057000) using the microscope's camera (PDV 010-82613119) attached to a personal computer (PC). Counting of cells positive for KI-67 was done and quantified using ImageJ software.

Statistical Analysis

Data obtained from this investigation were analyzed using Graphpad Prism statistical software (version 8.0.2). Analysis of Variance (ANOVA) was used to compare mean between groups. All data were expressed as mean ± standard error of mean (S.E.M) at $p < 0.05$.

RESULTS

Effect of *Uvaria chamae* on Oxidative Stress Markers following Cadmium Chloride-induced Prostate Pre-malignancy

The results presented below showed a significant and a dose-dependent increase in the prostate tissue levels of SOD, CAT and GPx in group 4, 5 and 6 animals administered with 3mg/kg of cadmium chloride followed by 2500 mg/kg, 1500 mg/kg and 1000 mg/kg of *Uvaria chamae* root extract for 28 days respectively when compared to control and other groups ($p < 0.05$). Moreso, there was a significant decrease in the prostate tissue levels of SOD, CAT and a significant increase in the prostate tissue levels of MDA and GPx in the cadmium chloride alone group given 3 mg/kg of cadmium chloride for 28 days when compared to control and other groups.

Expression of Ki-67

Low Ki-67 expression was evident in the control group (figure 5). A well differentiated Ki-67 expression with a percentage positive cells of about 22 % was noted in the cadmium chloride alone group (figure 6). Ki-67 stain was moderately expressed in group 3 (given 3 mg/kg of $CdCl_2$ plus 150 mg/kg of casodex for 28 days) and the percentage of positive cells was quantified to be 8 % when compared to control (figure 7). A poor expression was evident in group 4 (given 3 mg/kg of $CdCl_2$ plus 2500 mg/kg of *Uvaria chamae* root extract for 28 days respectively) and a decrease in the percentage of positive cells (7 %) (figure 8). Percentage of positive cells was quantified to be 7 % in group 5 (given 3 mg/kg of $CdCl_2$ plus 1500mg/kg of *Uvaria chamae* root extract for 28 days respectively) with poorly differentiated Ki-67 positivity (figure 9). A moderately differentiated K1-67 and percentage of positive cells of 10 % was evident in group 6 (given 3 mg/kg of $CdCl_2$ plus 1000mg/kg of *Uvaria chamae* root extract for 28 days respectively) (figure 10). There were poor expressions of Ki-67 expressivity in the casodex and the various doses of *Uvaria chamae* alone treated tissue sections respectively (figures 11, 12, 13 and 14).

DISCUSSION

For many centuries, medicinal plants have been reported to contain substances of therapeutic importance for the treatment of many forms of ailments and diseases [10]. Oxidative stress denotes an imbalance between oxidants' production and an organism's defense system [11] and is widely associated with various forms of inflammatory and metabolic diseases, including carcinogenesis [12-13]. It is correlated with damage cumulatively caused by free radicals that are inadequately neutralized by antioxidants [14]. Free radicals have been documented to adversely affect the survival of a cell due to membrane damage as a result of irreversible DNA modification, oxidative damage of protein and lipid. Oxidants can be produced through different means such as penetrating radiations, enzymatic reactions and chemical reactions [15]. Oxidative stress is closely associated and related to all forms of pre-malignant and malignant conditions. When the capacity of oxidation-reduction system in the body is exceeded by oxidative stress, it results in gene mutations, adverse effect on intracellular signal transduction and transcription factors resulting in carcinogenesis [16]. From our findings, catalase and superoxide dismutase were observed to be significantly decreased in cadmium chloride-induced group administered with 3mg/kg of cadmium chloride intraperitoneally but together with gluthathione peroxidase dose-dependently elevated in the prostate tissues of rats given 3mg/kg of cadmium followed by 2500 mg/kg, 1500 mg/kg and 1000 mg/kg of *Uvaria chamae* root extract respectively. Catalase is one of the most important antioxidant enzymes that can attenuate oxidative stress in an organism by converting cellular hydrogen peroxide into water and oxygen [17]. The conversion involves a two-step reaction. Decrease in catalase activity as witnessed in our findings is associated with a wide range of diseases such as cancer, diabetes mellitus and heart diseases. Administration of 2500 mg/kg of *Uvaria chamae* root extract was able to potentiate increased catatase activity which is associated with the prime role of regulating the cellular level of hydrogen peroxide. Catabolism of hydrogen peroxide protects cellular components from assault caused by oxidative stress and has been implicated to act as cellular messenger. Catalase can be used as a therapeutic agent against numerous oxidative stress related diseases [18]. Superoxide dismustase acts against reactive oxygen species-mediated diseases. It plays a critical role in hindering oxidative inactivation of nitric oxide thus preventing mitochondrial dysfunction [19]. SOD catalyses the dismutation of oxygen ion into oxygen molecule and hydrogen peroxide, this process requires a catalytic metal at the site of the enzyme [20]. The root extract of *Uvaria chamae* is believed to initiate these catalytic processes by causing an alternate reduction and re-oxidation of redox active transition metals such as copper and manganese. Decrease in SOD activity as witnessed in cadmium chloride-induced group has been implicated in the pathogenesis of many diseases such as cancer, heart diseases and diabetes and has been related to the destructive effect of oxygen ion superoxide which contributes to the increased rate of

phosphorylation in numerous oncogenic signaling processes. Numerous studies have documented that, decreased enzymatic activity of SOD is evident in all types of cancer including prostate cancer [21]. Gluthathione peroxidase is a selenium dependent enzyme and can be found in cytosol, mitochondria and extracellular fluid [22]. Unlike catalase, it reduces organic hydroperoxides and neutralizes hydrogen peroxide using gluthathione monomer as a proton donor cofactor. Increased levels of GPx were dose-dependently observed in all treatment groups (2500 mg/kg, 1500 mg/kg and 1000 mg/kg root extracts of *Uvaria chamae* respectively) and the cadmium chloride-induced group compared with control. This is due to the fact that carcinogenesis has greater level of oxidative stress and by so doing, the body increases the anti-oxidant system level in order to compensate for the increased levels of reactive oxygen species as a natural protective and defense mechanism against cancer [23]. Our finding is in agreement with numerous other reports which showed increase in Gpx levels in many tumour cells including squamous cell carcinoma [24], breast [25], lung [26], brain [27] and colorectal tumours [28] when compared with healthy controls. Our findings showed the lack of Gpx defense system followed by increased oxygen species resulting in the development of pathological prostate conditions. MDA was significantly increased in cadmium chloride-induced group when compared with control and other groups. This increase is due to the production of major by-product generated by lipid peroxidation. The by-products formed in cells modify proteins by reacting with compounds having a thiol group [29]. MDA can form stable compounds with amine groups of proteins, phospholipids and nucleic acid to form both intra and inter-molecular bridges of amino-3-iminopropene with structural modifications, eventually binding to the nucleus of a cell. At this point, MDA binds with nucleotide bases, cytosine and quinine to form bridges between the DNA strands thereby inducing mutagenic tendency [30]. Adding to *Uvaria chamae*'s reducing scavenging capacity, the root extract was found to stimulate the levels of many anti-oxidative enzymes such as CAT, SOD and GPx but significantly down-regulated MDA level. Increase in the levels of anti-oxidant enzymes after treatment with *Uvaria chamae* can be associated with the presence of polyphenols and others in the plant. Polyphenols are able to demonstrate their antioxidant activity via two major pathways which are; acting as radical scavengers to prevent the cellular damage caused by reactive oxygen species and acting as molecules that inhibit the production of ROS [31]. Polyphenols can also inhibit ROS via their metal chelator activity that involves the availability of iron [31]. The bioactive agents in the root of *Uvaria chamae* was realized to inhibit cancer cell proliferation and decrease oxidative stress in a time and dose-dependent manner and thus can open new dimensions for prostate cancer management and therapy. Furthermore, with the growing knowledge of molecular and cancer biology, several prognostic approaches have been used to evaluate tumour proliferation. In this study, Ki-67 was evaluated to ascertain tumour cell proliferation. Ki-67 is a nuclear protein associated with proliferation of cells and is encoded by the MK167 gene [32]. Well-differentiated Ki-67 expression was observed in the cadmium chloride alone treated group and its expression is documented and reported to be expressed in certain cell cycle phases which include S-phase, G-phase and the M-phase but does not exist in the G0 phase [33]. It is worthy to note that ki-67 positivity portrays a higher risk of cancer occurrence and a decline or worse survival rates in patients with early prostate cancer, thus a high expression of this nuclear protein can be associated with worse prognosis [34] and a ki-67 labeling index is the percentage of cells with ki-67 positivity. Quantification of positive cells was done using image analysis technique, 22 % of positive cells were noted using ImageJ software in the cadmium chloride alone group. Ki-67 has been considered a biomarker for therapeutic decision [35]. [36] documented the role of proliferation marker as a predictive tool in early cancer. This study is in agreement with [37] who reported that ki-67 was significantly up-regulated in 64% of prostate cancer. Also [38] and [39] reported an increased expression of ki-67 marker in prostate cancer with high tumour grading. Ki-67 is documented to be up-regulated in prostate cancer but increased expression is notably found in aggressive and high grade

tumour of the prostate. Expression of Ki-67 is associated with cell proliferation status. Over-expression of Ki-67 results in the formation of heterochromatin and its involvement in the organization of chromatin was demonstrated by an investigation which showed that the Ki-67 protein is needed to maintain heterochromatin at inactive chromosome X in non-transformed cells. Ki-67 expression was dose-dependently down-regulated in all the *Uvaria chamae* treated animals when compared to control and standard. Poorly differentiated Ki-67 expressions were noticed with subsequent decrease in the number of stained cells and areas after image analysis technique in the *Uvaria chamae* treated animals. The percentage stained cells observed in all the *Uvaria chamae* treated group were 7 %, 7 % and 10 % respectively when compared to the cadmium chloride alone sections with 22 % and standard with 8% respectively. The Ki-67 index which is the percentage of tumour cells positive for Ki-67 staining strongly correlates with the S-phase of the cell cycle. During the S-phase, DNA duplication takes place. The down-regulation of Ki-67 is due to the anti-tumour activity of the root extract of *Uvaria chamae* which resulted in slower tumour growth. The bioactive compounds present in the root are believed to enhance the G0 phase of the cell cycle and during this phase, Kis-67 is down-regulated [32]. Similarly, Ki-67 protein down-regulation also occurs in the G1 phase through the ubiquitin proteasome complex. The root extract of the plant is believed to stimulate this complex and thus expression of ki-67 can significantly predict the outcome of prostate cancer. *Uvaria chamae* root extract can be considered a potent and harmless chemotherapy agent since many chemotherapeutic drugs act to break the DNA strands to stop cell replication [40].

CONCLUSION

Results obtained from this study showed that ethanol root extract of *U. chamae* down-regulated tissue level of MDA and KI-67 expression but up-regulated tissue levels of SOD, CAT and GPx in cadmium chloride-induced prostate pre-malignancy. Furthermore, our study is novel and showed that *U. chamae* root exhibit strong anti-cancer property in prostate pre-malignancy especially at 2500 mg/kg and 1500mg/kg.

ACKNOWLEGDEMENT

Special thanks to Tertiary Education Trust Fund (TETFUND) for supporting and sponsoring this research.

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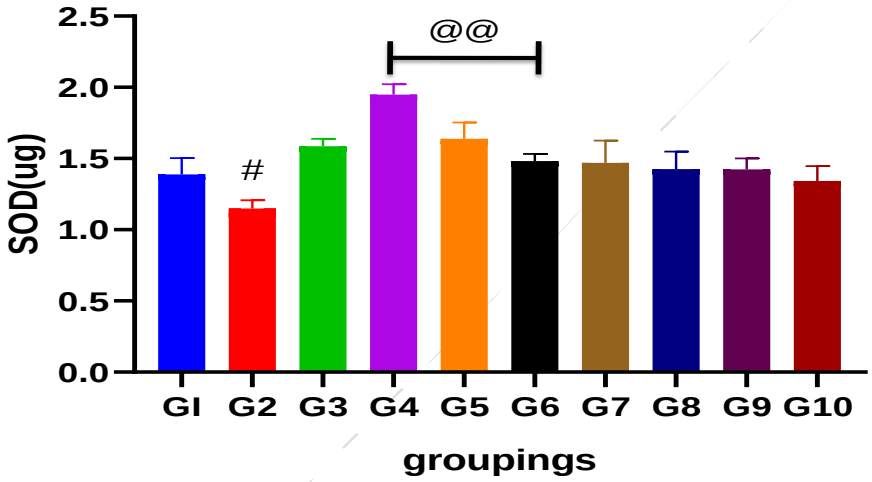

Figure 1: Chart indicating tissue SOD level following cadmium chloride-induced prostate pre-malignancy.
= Significantly different from control and other treated groups
@@ = Significantly different from group 2

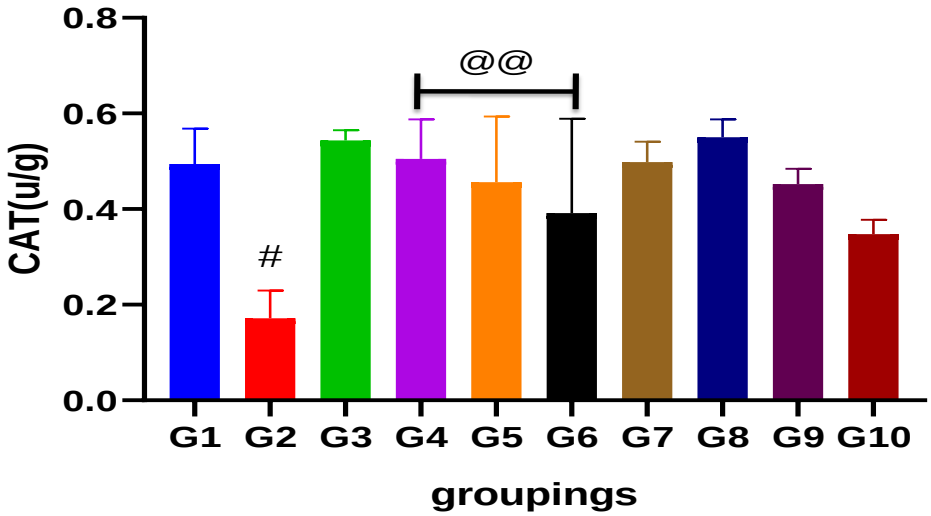

Figure 2: Chart indicating tissue CAT level following cadmium chloride-induced prostate pre-malignancy.
= Significantly different from control and other treated groups
@@ = Significantly different from group 2

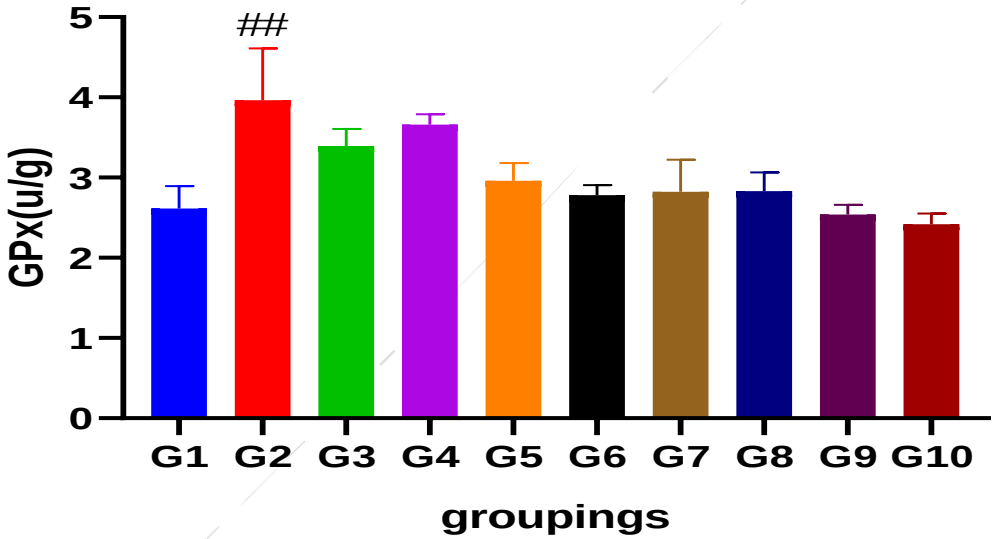

Figure 3: Chart indicating tissue GPx level following cadmium chloride-induced prostate pre-malignancy.
= Significantly different from control

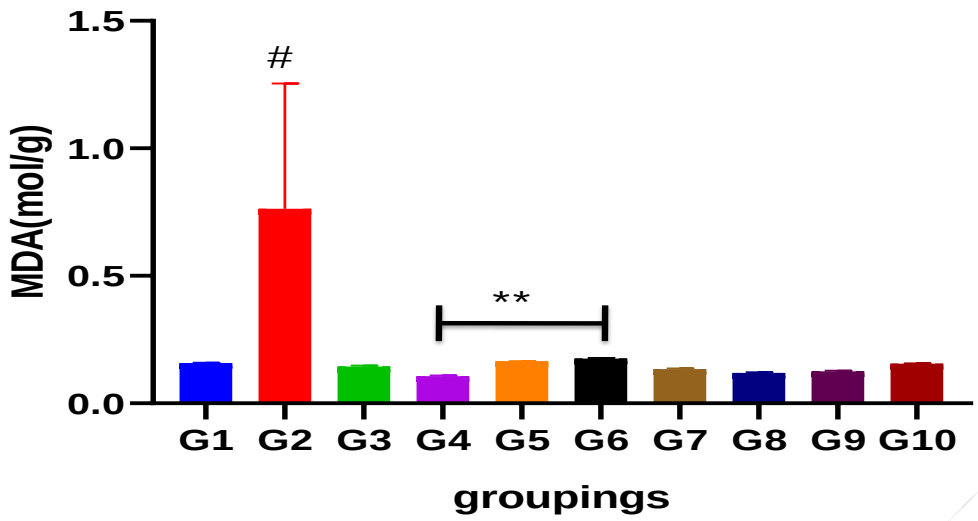

Figure 4: Chart indicating tissue MDA level following cadmium chloride-induced prostate pre-malignancy.
= Significantly different from control and other treated groups
** = Significantly different from group 2

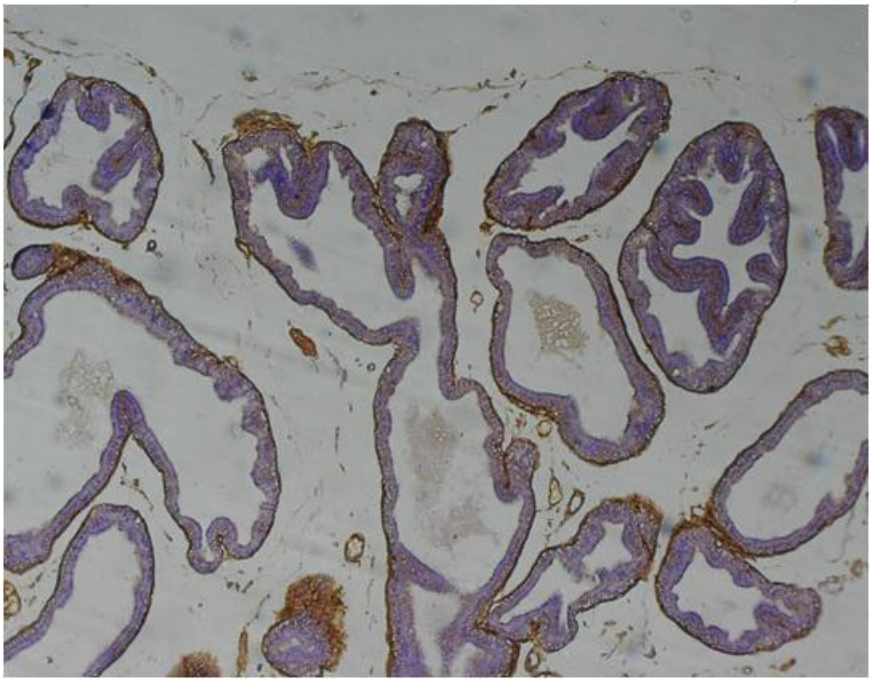

Figure 5: Photomicrograph of control given feed and water showing poor Ki-67 expression (+). % of positive cells= 1%. Ki-67 x100 magnification.

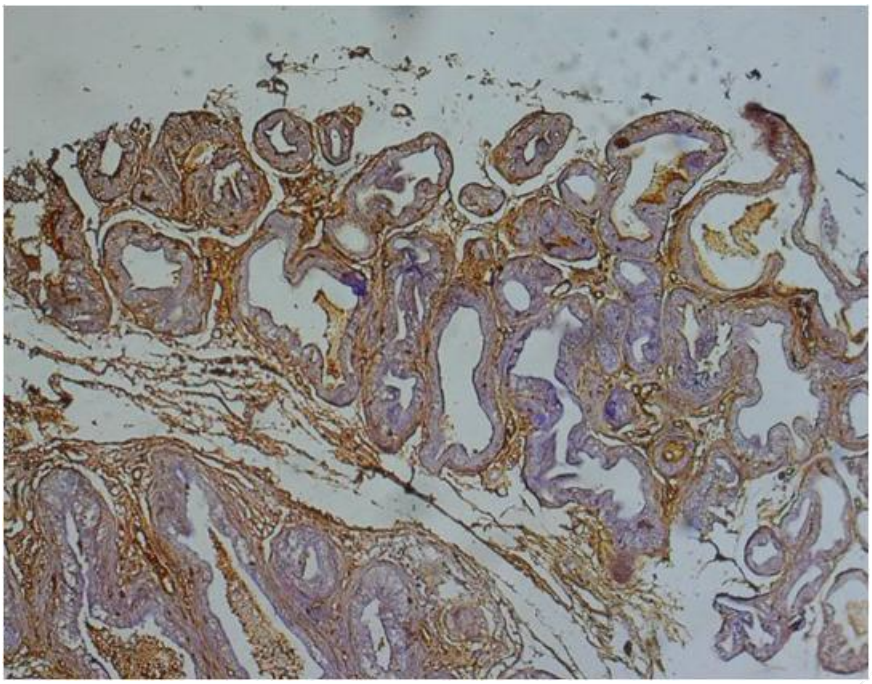

Figure 6: Photomicrograph of group 2 rats given 3mg/kg of cadmium chloride alone showing well-differentiated Ki-67 expression (+++). % of positive cells= 22%. Ki-67 x100 magnification.

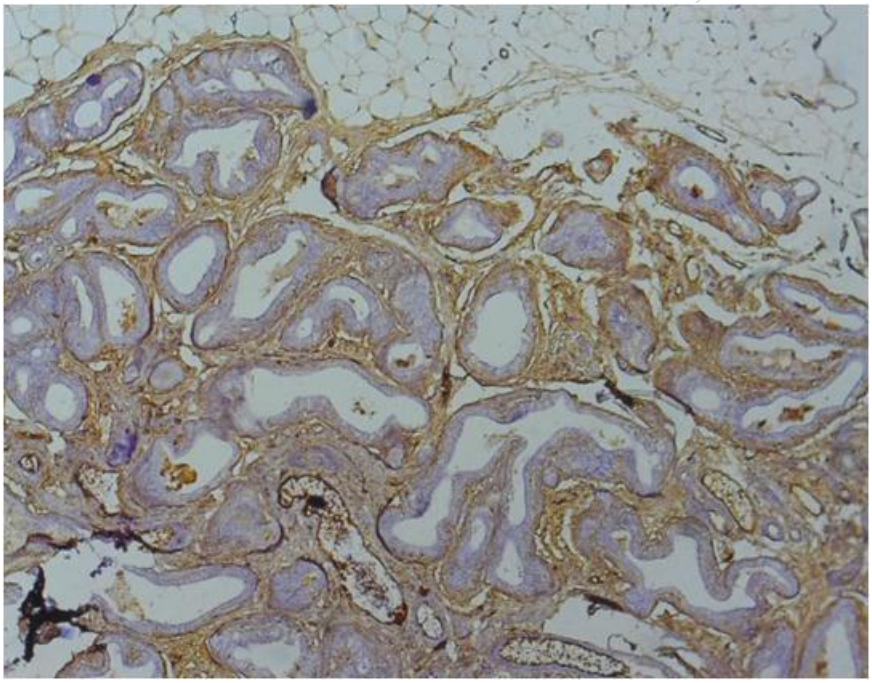

Figure 7: Photomicrograph of group 3 rats given 3mg/kg of cadmium chloride and 150mg/kg casodex showing moderately differentiated Ki-67 positivity (++). % of positive cells =8%. Ki-67 x100 magnification.

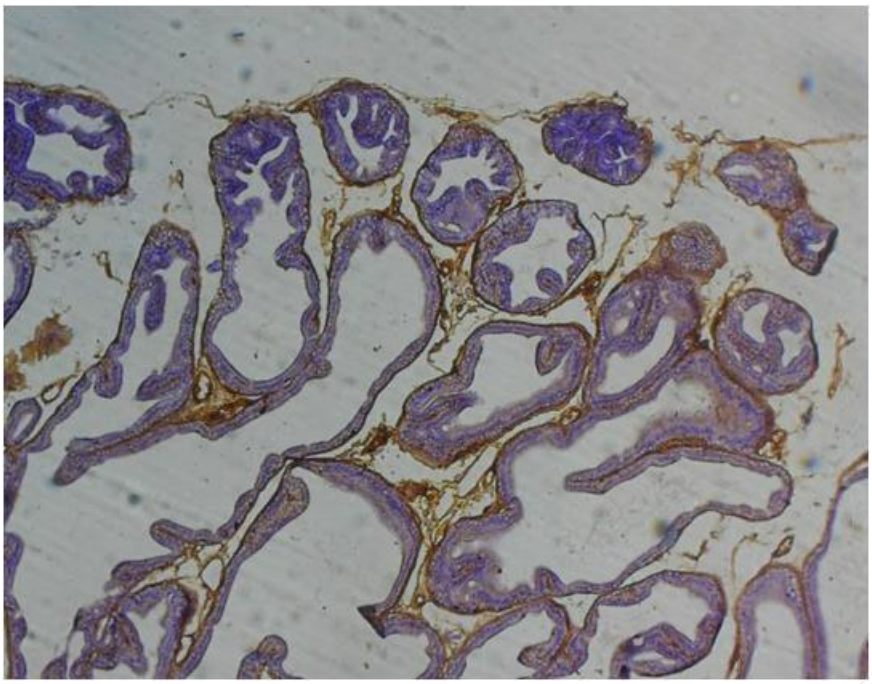

Figure 8: Photomicrograph of group 4 given 3mg/kg of cadmium chloride and 2500mg/kg of *Uvaria chamae* root extract showing poorly differentiated Ki-67 positivity (+). % of positive cells =7%. Ki-67 x100 magnification.

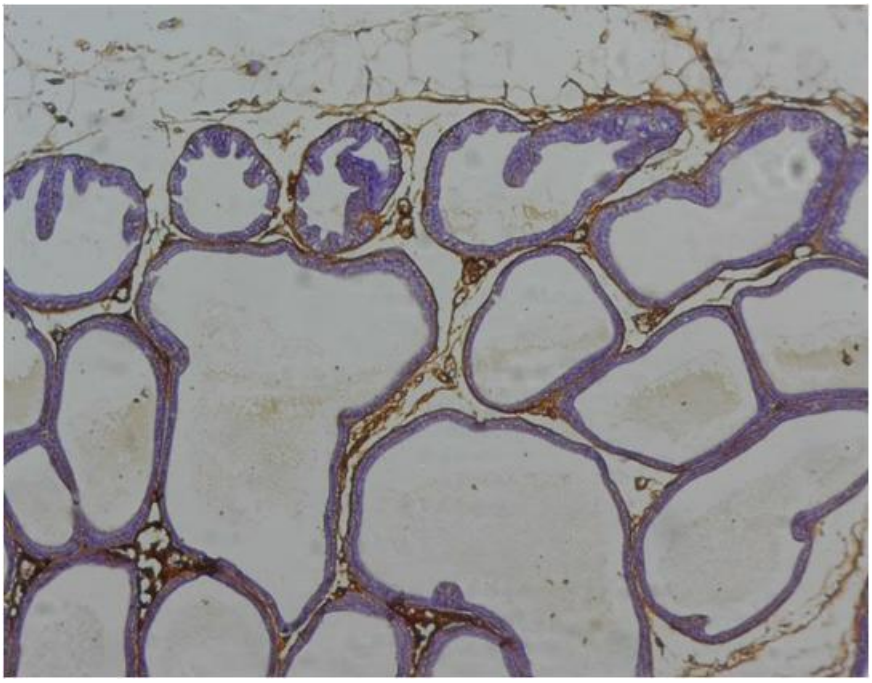

Figure 9: Photomicrograph of group 5 rats given 3mg/kg of cadmium chloride and 1500mg/kg of *Uvaria chamae* root extract showing poorly differentiated Ki-67 positivity (+). % of positive cells =7%. Ki-67 x100 magnification.

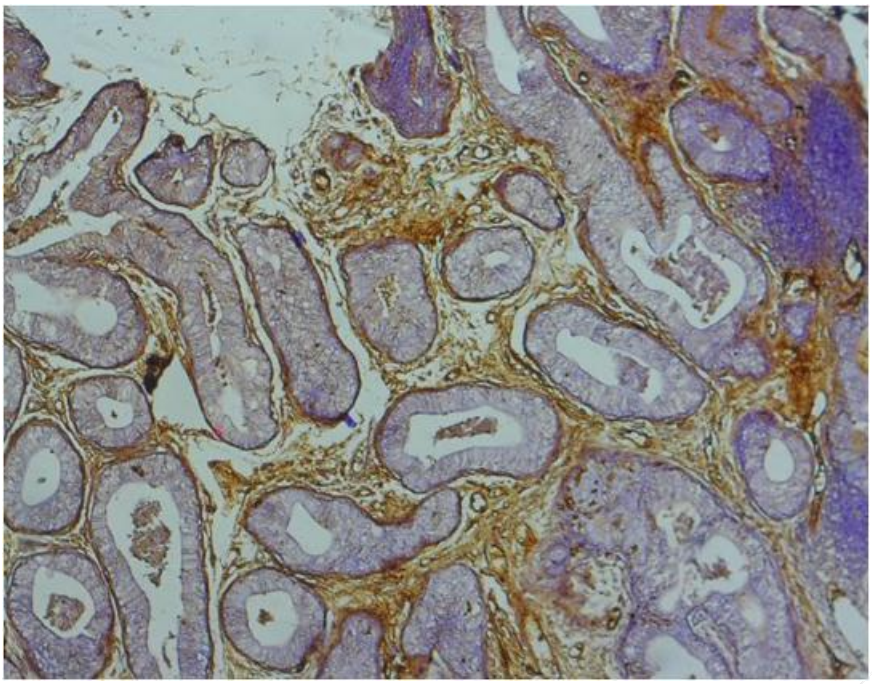

Figure 10: Photomicrograph of group 6 rats given 3mg/kg of cadmium chloride and 1000mg/kg of *Uvaria chamae* root extract showing moderately differentiated Ki-67 positivity (++). % of positive cells =10%. Ki-67 x100 magnification.

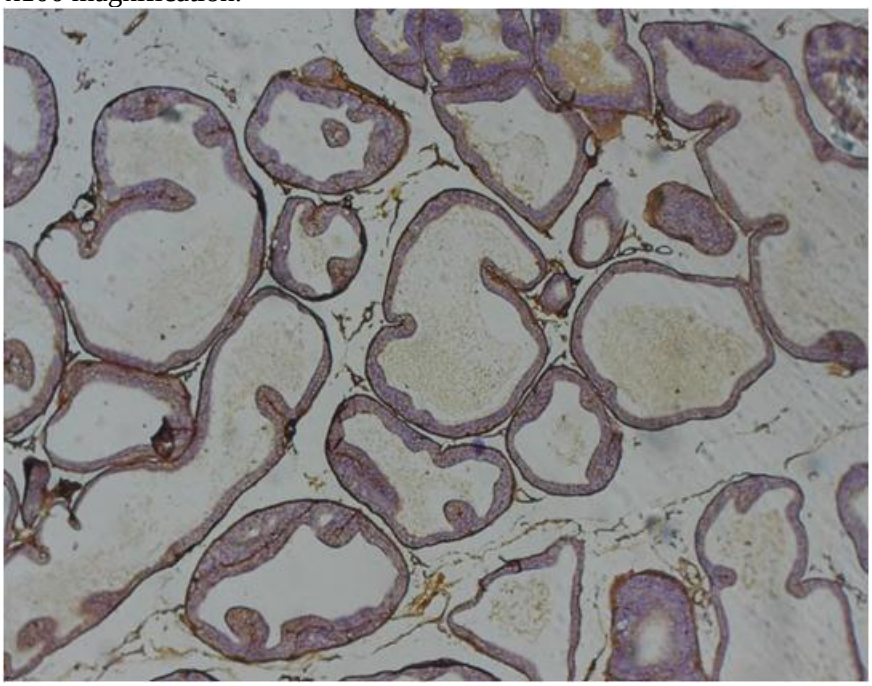

Figure 11: Photomicrograph of group 7 rats given 150mg/kg of casodex alone showing poor expression of Ki-67 (+). % of positive cells = 1%. Ki-67 x100 magnification.

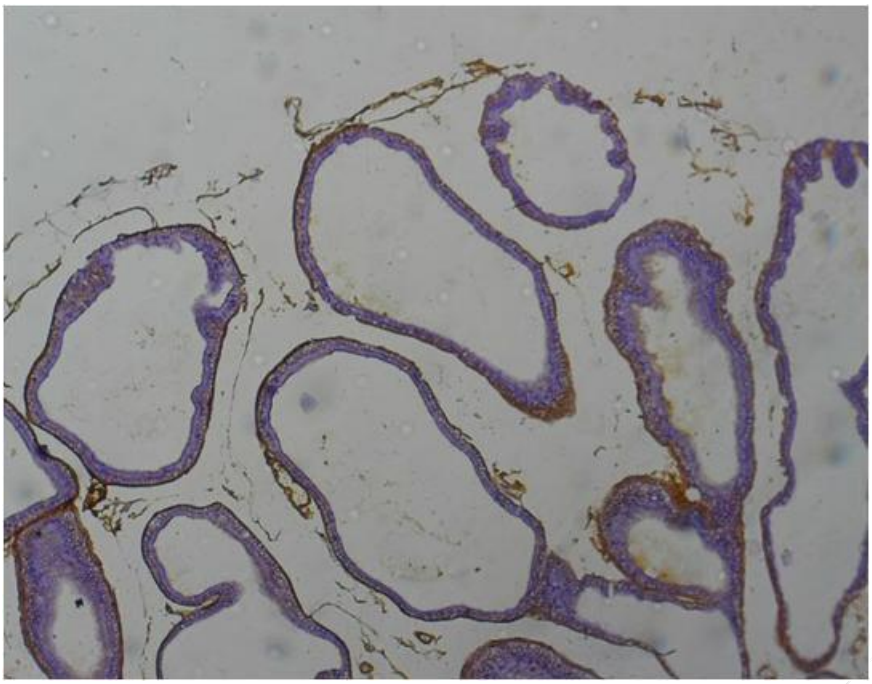

Figure 12: Photomicrograph of group 8 rats given 2500mg/kg of *Uvaria chamae* root extract alone showing poor expression of Ki-67 (+). % of positive cells = 1%. Ki-67 x100 magnification.

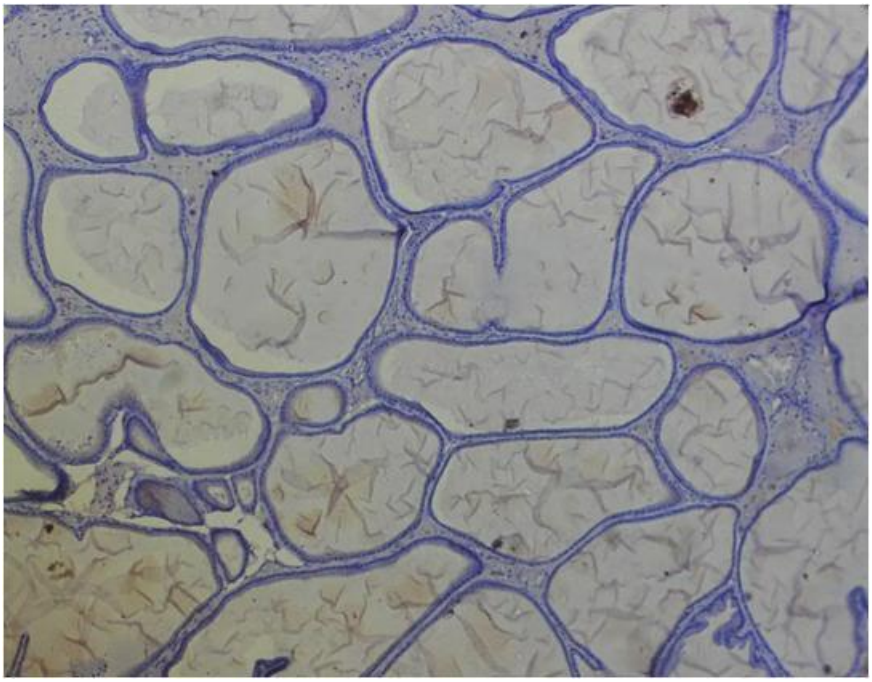

Figure 13: Photomicrograph of group 9 rats given 1500mg/kg of *Uvaria chamae* root extract alone showing poor expression of Ki-67 (+). % of positive cells =1%, Ki-67 x100 magnification.

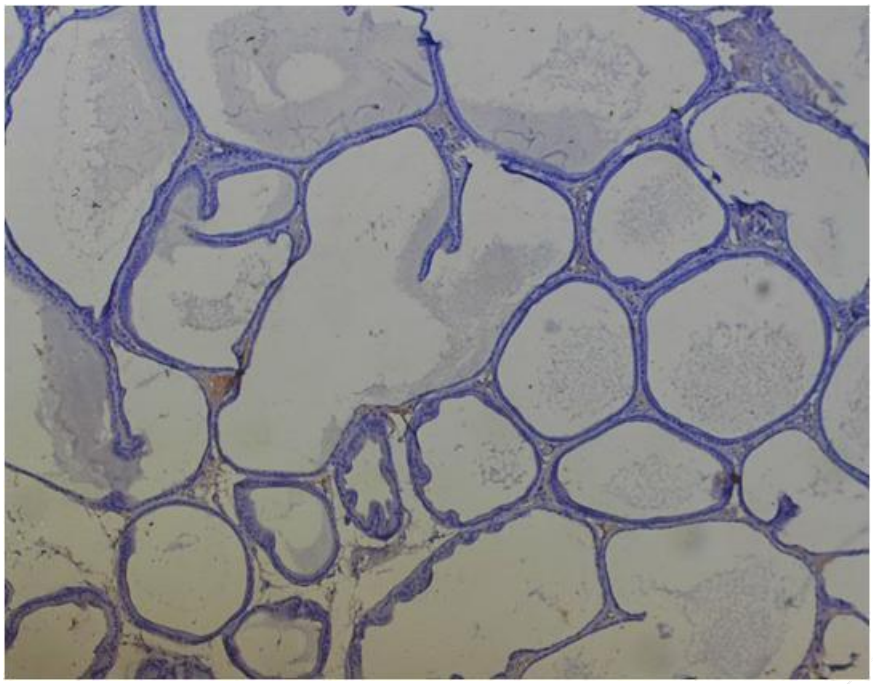

Figure 14: Photomicrograph of group 10 rats given 1000mg/kg of *Uvaria chamae* root extract alone showing poor expression of Ki-67 (+) % of positive cells = 1%. Ki-67 x100 magnification.