Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2021; 13(4);12-21
ISSN: 0975-4873

Research Article

Carcinogenic and Mutagenic Effects of Betel Nut with Haematological, Histopathological and Cytological Toxicity in Solid Tumour Bearing Mouse

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Received: 19th Nov, 21; Revised 4th December, 21, Accepted: 14thDecember, 21; Available Online:25th December, 21

ABSTRACT

The aim of the present study is to evaluate how ethanolic betel nut extract (BNE) at different doses influences the tumour growth rate on solid tumour bearing Swiss albino mouse along with haematological, histopathological and cytogenetical toxicity. In addition, the nature of vascular density of tumour has also been studied morphometrically to investigate the angiogenic effect of BNE. A solid tumour was induced on leg for experiment. After five days of tumour cell inoculation ethanolic BNE was injected intraperitoneally on alternating days. The solid tumour growth gradually increased with the steady increase of tumour vascularisation with increasing concentrations of BNE treatment. A steady decrease in the haemoglobin percentage and total RBC count along with lymphocyte population was noted with the steady increase in the WBC count and neutrophil population. Moreover, BNE at the different concentrations induces significant genetic damage i.e. chromosomal aberration in bone marrow cells and histopathological abnormalities in liver in solid tumour bearing mice. Therefore, our present studies indicate that ethanolic betel nut extract causes significantly mutagenic, carcinogenic and angiogenic effects on solid tumour bearing mice.

Keywords: Betel nut extract,Sarcoma 180, Solid tumour, RBC, WBC, Chromosome, Angiogenesis

INTRODUCTION

The growth of new blood vessels from the pre-existing vasculature is the main driving force in initiation and development of tumour and determines the metastatic potential. The blood vessel supports the tumour growth by suppling passage of oxygen and nutrients.[1,2] Epidemiological studies revealed that consumption of smoked fish, meat, wild mushroom and different types of plant products such as leaves and seeds of *Lathyrus sativus* are associated with different types of diseases including cancer.[3, 4] So, the potential hazard of its wide application cannot be ignored. The experiments since the last few decades have generated evidence that betel nut acts as a genotoxic and carcinogenic agent in animals as well as humans. Earlier studies have been carried out to know the genotoxic and cytotoxic potential of BNE on different in vivo and in vitro cell systems considering chromosome aberration and SCE studies.[5] Our preliminary studies revealed that BNE plays a substantial role in the development of solid tumour growth in mouse which was analysed morphometrically.[6] Therefore, the present study has been oriented to carry out a thorough analysis on solid tumour in Swiss albino male mice in response to BNE considering the growth rate with tumour vasculature, haematological toxicity, histopathological particularly hepatotoxicity and cytological toxicity for better understanding of mutagenic, carcinogenic and angiogenic effect.

MATERIALS AND METHODS

Collection and Extraction of betel nut

Ethanolic betel nut was prepared with some modifications of the methodology originally described by earlier researchers.[3,7,8] Fresh betel nut was purchased and authenticated by Botanical survey of India, Botanical Garden, Howrah, West Bengal, India.100gm of betel nuts were processed into small pieces and kept in 90% ethanol overnight for extraction. The solution of betel nut was then placed into the thimble of a soxhlet and the extraction process was continued for 48 hours. The ethanolic solution was filtered twice and dried in an incubator at 50°C. Then the dried mass of BNE was kept for future use.

Experimental animals

Nine-week-old healthy Swiss male albino mice (*Mus musculus*) and weighing about 20gm were used for the present experiment. The mice were acclimatized in the experimental room under controlled condition of temperature (24ºC - 26 ºC) and humidity. The animals were fed sterilised food pellets and water *ad libitum*. The experiments were carried out in accordance with the rules and guidelines formulated by the Institutional Animal Ethics Committee (Animal House Registration No. – 1795 / PO / Ere / S/ 14 CPCSEA- 31/12/2014), Rammohan College, Kolkata, for maintenance and care of laboratory animals.

Selection of animal tumour model and tumour transplantation

Sarcoma 180 (S-180) murine ascitic tumour cell lines were maintained *in vivo* by serial intraperitoneal transplantation in the laboratory. The solid tumour was generated by

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injecting 1 x 10^6 ascitic tumour cells (0.4 ml) subcutaneously in the leg muscle of normal mouse. A palpable tumour was visualized and the treatment was started on 6th day of tumour cell injection.

Preparation of BNE solution and mode of administration

The BNE extract was prepared by dissolving it in sterilised distilled water. Three different concentrations of BNE such as 100 mg/ kg, 200 mg / kg and 300 mg / kg of body weight were prepared for treatment. Experimental animals were divided into five groups. Each solid tumour bearing mouse (treated group) received BNE intraperitoneally at a concentration of 100 mg or 200mg or 300mg / kg body weight on alternating days (Table – 1). A parallel positive control or vehicle was done with the administration of distilled water (1ml / 100 gm body weight) intraperitoneally. The tumour bearing mouse without any injection was treated as negative control.

Experiments on solid tumour

Morphometric analysis of solid tumour volume

The solid tumour was isolated after sacrificing mice and the images of tumour in control, vehicle and BNE treated mice were captured by camera (Sony cybershop) and percentage of area was measured by image J software (Version 1.53k).[9]

Haematological parameters

Percentage of Haemoglobin

Control and treated mice were sacrificed by cervical dislocation. Blood was drawn by a syringe from the heart of mouse and mixed with EDTA in a vial. Then the Hb gram percentage was determined in a Sahli's heamoglobinometer by following the method of Sood and Math *et al.*[10,11]

Total RBC and WBC count

Total RBC and WBC count were determined from peripheral blood of control and treated solid tumour bearing mouse according to the method of earlier researchers.[10,11,12]

Differential count of neutrophils and lymphocytes

Differential counts of neutrophils and lymphocytes were evaluated from Leishman-stained blood cells of control and treated mice according to the method of Sood.[10, 13]

Histopathological analysis of liver

Liver of control and treated mouse were excised into small pieces and fixed in 10% neutral buffered formalin for 24 hours. The fixed tissues were washed in tap water, dehydrated and processed for paraffin block sectioning. 5 µm thin mounted tissue sections were then deparaffinized and stained with hematoxylin and eosin following the standard procedure.[14, 15] Histopathological analysis was done by light microscopy to evaluate the nature and extent of lesion and damage in liver tissue.

Study of Chromosome aberration to determine mutagenicity from bone marrow cells

Somatic metaphase chromosome was prepared from bone marrow cells of control and treated mouse by a slight modification of the original technique of Chakrabarti *et al.*[3] The technique is described thus:

Animals received injection of 0.04% of colchicine (Sigma, St Louis, U.S.A.) solution intraperitoneally at a rate of 1 ml /100 gm. body weight 1.5 hours prior to sacrifice. Cells from the bone marrow were collected in a hypotonic solution of 0.075 M KCl and aspirated to make milky solution. Then the solution was incubated at 37ºC for 25 minutes and centrifuged at 1500 rpm for 15 minutes. Supernatant was discarded and cell pellet was fixed in aceto alcohol fixative. The fixative was prepared from methanol and glacial acetic acid in 3:1 proportion. Aceto alcohol fixed bone marrow cell solution was again centrifuged for 10 minutes and the cell sediment was fixed in aceto alcohol. Three drops of cell suspension were dropped on clean 50 % ethyl alcohol-soaked chilled glass slides and allowed to dry in flame. The chromosome prepared slides were stained in 5% phosphate buffered Giemsa stain (pH 6.8) for 50 minutes. After washing, slides were observed under a binocular research microscope (10 × 100 magnifications) to analyse chromosomal abnormalities.[16, 17]

Analysis of blood vessels from the solid tumour

Tissues of solid tumour of control and treated mouse were excised with sharp scalpel and kept in 0.9 % NaCl solution for 2 minutes at room temperature. The tissues were mounted in glycerine and observed under a binocular research microscope at 5 x 10 magnifications to study the tumour vasculature according to the method practised in laboratory.[18] The nature of blood vessel of solid tumour of control and treated mouse was analysed by automated software (AngioTool v 0.6a) as standardized in the laboratory according to the method of earlier researchers.[19, 20, 21]

Statistical analysis

All data were expressed as a mean ± SE (n=3) and were statistically analysed by Student's *t* test.[22] Its significance was indicated by an asterisk. Morphometric analysis of tumour is evaluated by a specific formula as mentioned in the material and method section. Angiogenesis of tumour sample was analysed by Angio tool software.[19,20,21] The Flow diagram was created with Bio Render software (BioRender.com) and clip art image smart.servier.com. Angiogenesis Servier Medical art.[23,24]

RESULTS

Morphometric analysis of solid tumour volume

A steady increase in tumour growth was noted after the treatment of different concentrations of BNE (100mg/kg, 200mg/kg, and 300mg/kg body weight) as shown in the Figure 1A– E. The total area of tumour was significantly(**p>0.001) increased in all treated groups with increasing concentration of BNE (Figure 1F). The percentage of area was increased significantly (*p>0.05) in 100mg BNE/kg body weight treated mouse, but it was highly significant(**p>0.001) in 200mg BNE /kg and 300mg BNE /kg body weight treated mouse (Figure 1G).

Percentage of haemoglobin

The percentage of haemoglobin in BNE treated solid tumour bearing mouse decreased significantly(*p>0.05) in comparison to control and vehicle groups (Figure 2).

Total RBC and WBC count

The total count of RBC decreased and total count of WBC increased gradually with increasing concentrations of BNE

in all treated groups (Figure 3A and B). The rate of decrease of RBC count was more and highly significant (**p>0.001) in 200mg/kg and 300mg BNE/kg b. w. treated groups whereas the rate of increase of WBC count was more and highly significant (**p>0.001) in 200mg/kg and 300mg BNE /kg b. w. treated groups.

Differential count of neutrophils and lymphocytes

Neutrophil and lymphocyte populations were nearly equal in control and vehicle groups. But interestingly, an increase in neutrophil count and reduction in lymphocyte count (Figure 4A, B, C) were observed in all treated animals and were statistically significant (**p>0.001).

Table 1: Treatment schedule of control, vehicle, and treated groups of solid tumour bearing mouse. BNE treatment was started on 6th day of tumour cell injection and continued up to 10th day. Body weight (b. w.).

Treatment with concentration of BNE	Average weight of Mouse	Route of injection	Volume of injected solution
Negative control	20gm	-	-
Vehicle (sterilized distilled water injected because BNE was dissolved in it)	20gm	IP	1ml/100gm b. w
100 mg BNE /kg b. w. (low dose)	20gm	IP	1ml/100gm b. w
200 mg BNE /kg b. w. (medium dose)	20gm	IP	1ml/100gm b. w
300 mg BNE /kg b. w. (high dose)	20gm	IP	1ml/100gm b. w

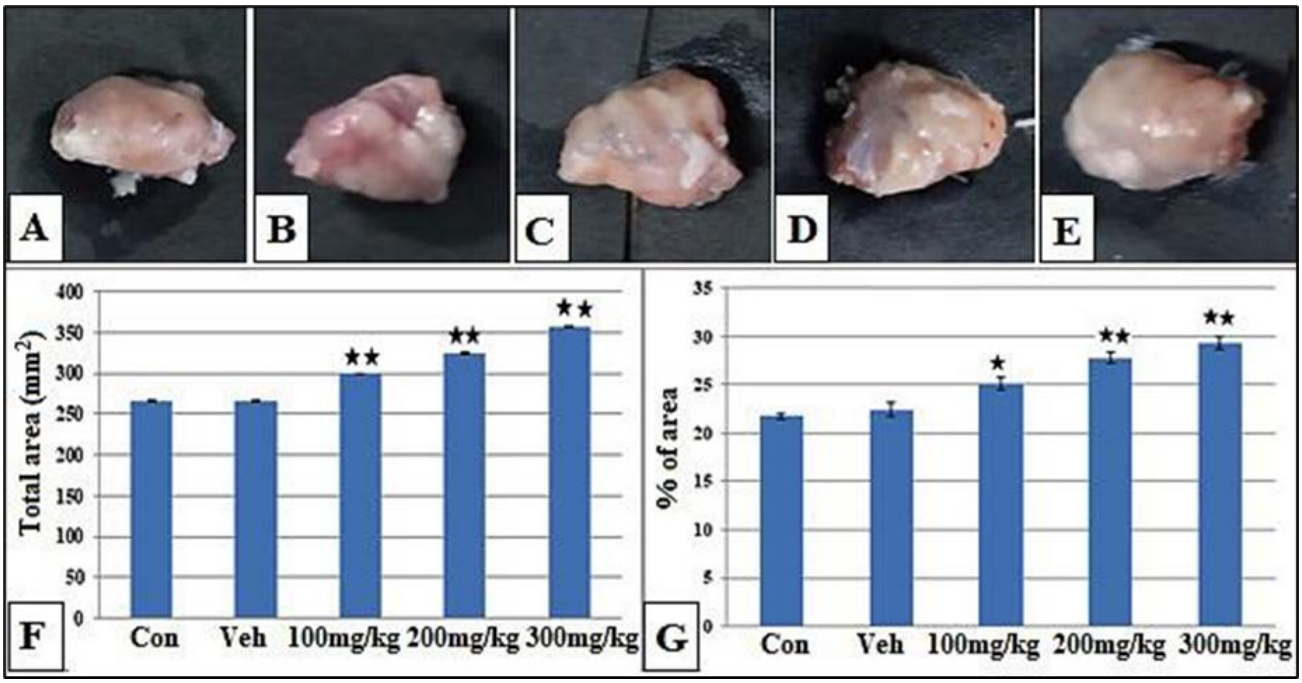


Figure 1: Determination of the impact of betel nut on the growth of solid tumour in mouse. Representative Image of Tumour growth of control (A), vehicle (B), 100mg/kg (C), 200mg/kg (D) and 300mg BNE /kg b. w.(E) treated mice. F: Graphical representation of total area of solid tumour of control, vehicle and treated series. Total area was significantly (**p>0.001) maximum in 300mgBNE /kg b. w. treated mouse. G. Graphical representation of % of area of control and treated series. In 300mg BNE /kg b. w. treated group, the percentage of area was significantly (**p>0.001) maximum than all groups.

Analysis of liver histology

Different types of histopathological abnormalities i.e., highly ruptured and congested central vein, abnormal vacuolated hepatocyte were observed in treated solid tumour bearing mouse (Figure 5A, B, C, D). The percentage of abnormalities of central vein (CV) was significantly (**p>0.001) increased in 200mg / kg and 300 mg BNE /kg b. w. treated animals (Figure 5E). The tissue area of control series was more when compared to the treated animals (Figure 5 F). Interestingly, the percentage of tissue area of 300mg BNE / kg b. w. treated animals was significantly (**p>0.001) very minimum.

The percentage of abnormal hepatocytes increased gradually with increasing concentration of BNE treatment which were highly significant (**p>0.001) in both 200mg and 300mg BNE / kg b. w. treated series (Figure 6). The abnormal hepatocytes were maximum in 300mg BNE / kg b. w. treated mouse.

Different types of plant products have been reported to induce somatic and germinal cell abnormalities as well as cancer development.[12, 13, 25,26,27,28] Stich (1991) advocated that high phenolic compound present in some plant extracts can cause different types of mutations and DNA strand breaks.[29] In this study, we investigated the potential carcinogenic effects of the betel nut extract on a solid

tumour bearing male mouse. The present study indicates that BNE has significantly enhanced the solid tumour growth when compared to that of the control. Tumour progression affected the haemoglobin percentage, total count of RBC and WBC and the differential count of neutrophil and lymphocyte. The haemoglobin percentage along with the total RBC count in treated animals was found to be reduced as compared to the control group. This may be due to haemolytic condition.[12, 13] The increasing population of neutrophil and decreasing population of lymphocyte in treated groups indicate toxicity on the haematological system of solid tumour bearing mouse.

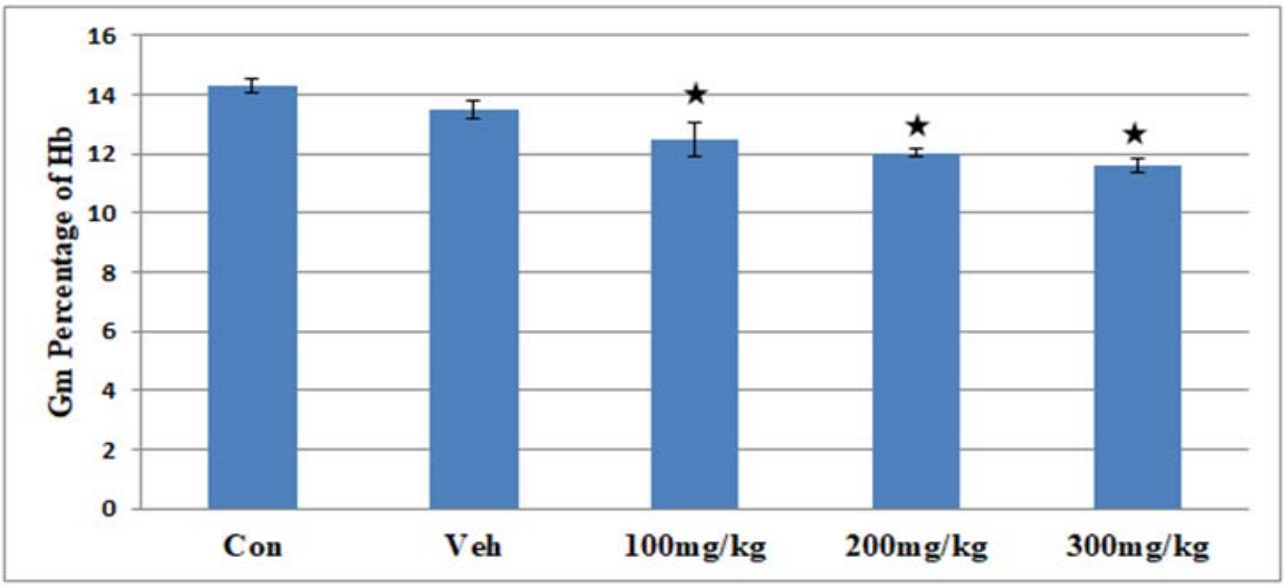


Figure 2: Graphical representation of Gram percentage of haemoglobin (Hb) in control (Con), vehicle (Veh) and treated groups. The Hb percentage significantly (*p>0.05) decreased in 100mg/kg, 200mg/kg and 300mg BNE /kg b. w. treated groups in comparison to control and vehicle Values are expressed as mean ± SE (n=3).

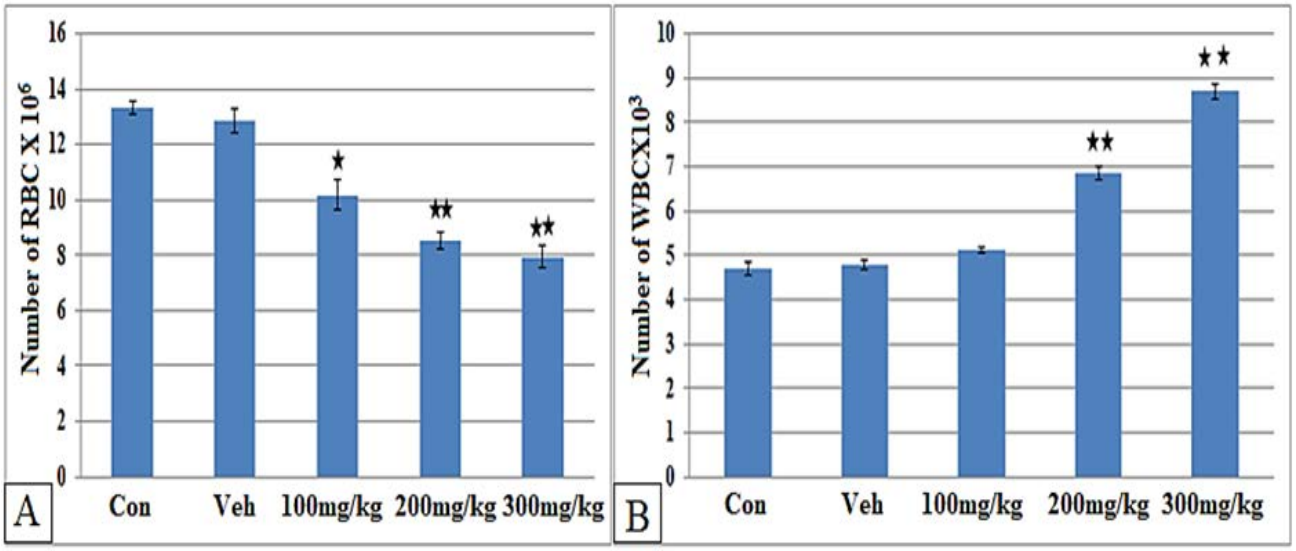


Figure 3: A. Graphical representation of total count of RBC in control (Con), vehicle (Veh)and treated groups. Total count of RBC decreased (*p>0.05) slightly in 100mg BNE / kg b. w. treated group but the rate of decrease was more and highly significant in both 200mg and 300mg BNE treated groups (**p>0.001). **B.** Graphical representation of total count of WBC in control (Con), vehicle (Veh) and treated groups. Total count of WBC increased in all treated groups but it was highly significant (**p>0.001) in 200mg BNE /kg and 300mg BNE / kg b. w. treated series. Values are expressed as mean ± SE (n=3).

Histopathological examination of liver sections in BNE treated group showed large areas of ruptured and congested central vein, abnormal vacuolated hepatocyte in comparison to the control group. Our previous works proved that ethanolic BNE contains high amount of phenolic components.[13] Therefore, the toxic phenolic compound may create an impact on hepatotoxicity.

Moreover, BNE induced significant mutagenic effects on bone marrow cells of treated mouse as is evident from the

studies of different types of structural chromosomal abnormalities and aneuploidy.

Furthermore, solid tumour bearing mice upon treatment with BNE showed increased angiogenesis in respect to the total number of blood vessels, vessel junctions and vessel end points when compared to the untreated control mice. Tazzyman et al (2013) advocated that neutrophil is the key mediator of tumour angiogenesis.[30] Neutrophils in malignant tumours, are able to release molecules that activate endothelial cells and promote angiogenesis.[31] The increasing population of neutrophil, evident in the BNE treated solid tumour bearing mice of our present study, may play an important role in the enhancement of tumour angiogenesis.

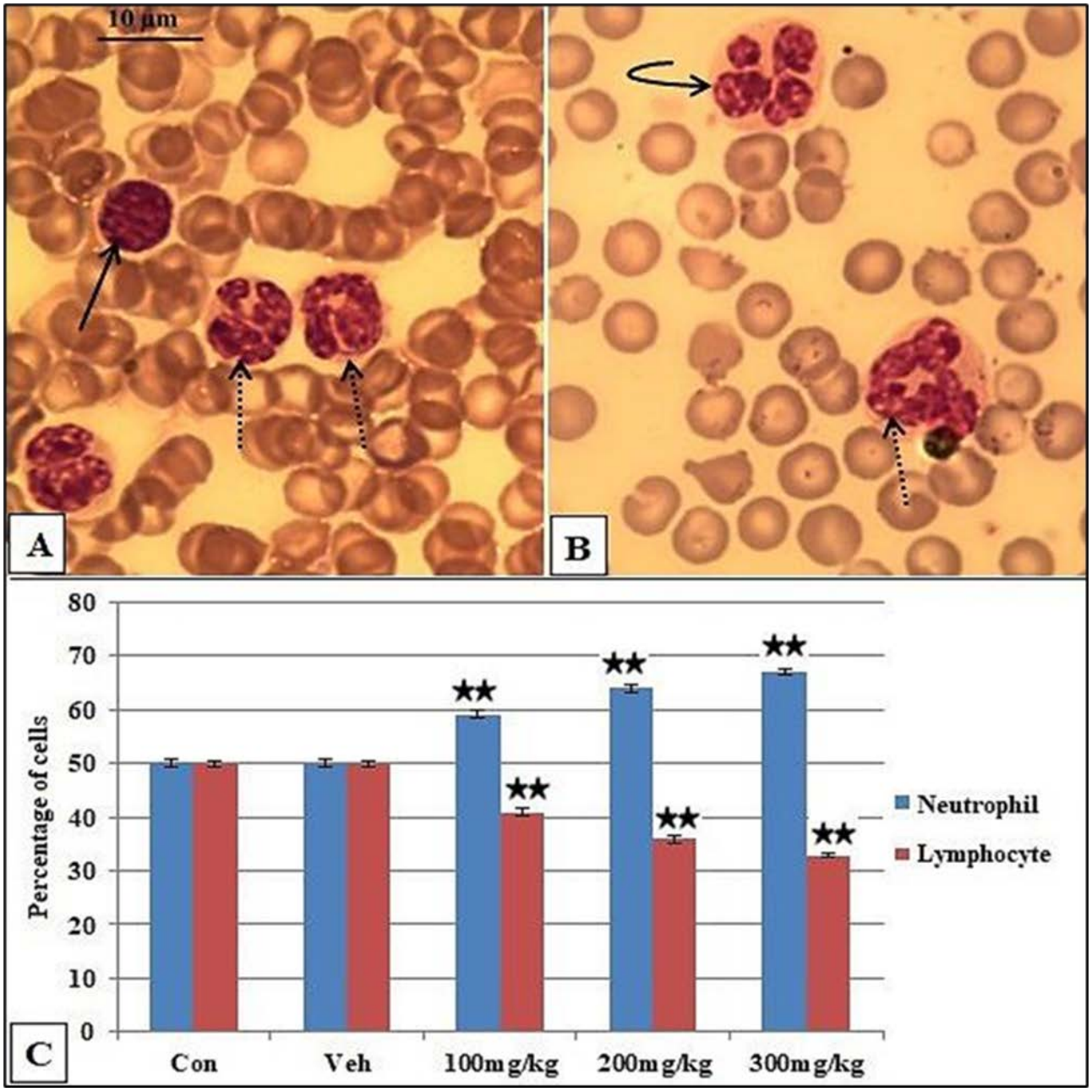


Figure 4: A. Leishman-stained blood film of BNE treated solid tumour bearing mice revealed lymphocyte (straight black arrow) and neutrophil (dotted arrow) population. The numbers of neutrophil were more than lymphocyte. B. Leishman-stained blood film of BNE treated solid tumour bearing mice revealed fragmented neutrophil (curved arrow). C. Graphical representation revealed that neutrophils increased and lymphocytes decreased in 100mg BNE /kg, 200mg BNE /kg and 300mg BNE / kg b. w. treated groups in comparison to control and vehicle (**p>0.001). Values are expressed as mean ± SE (n=3).

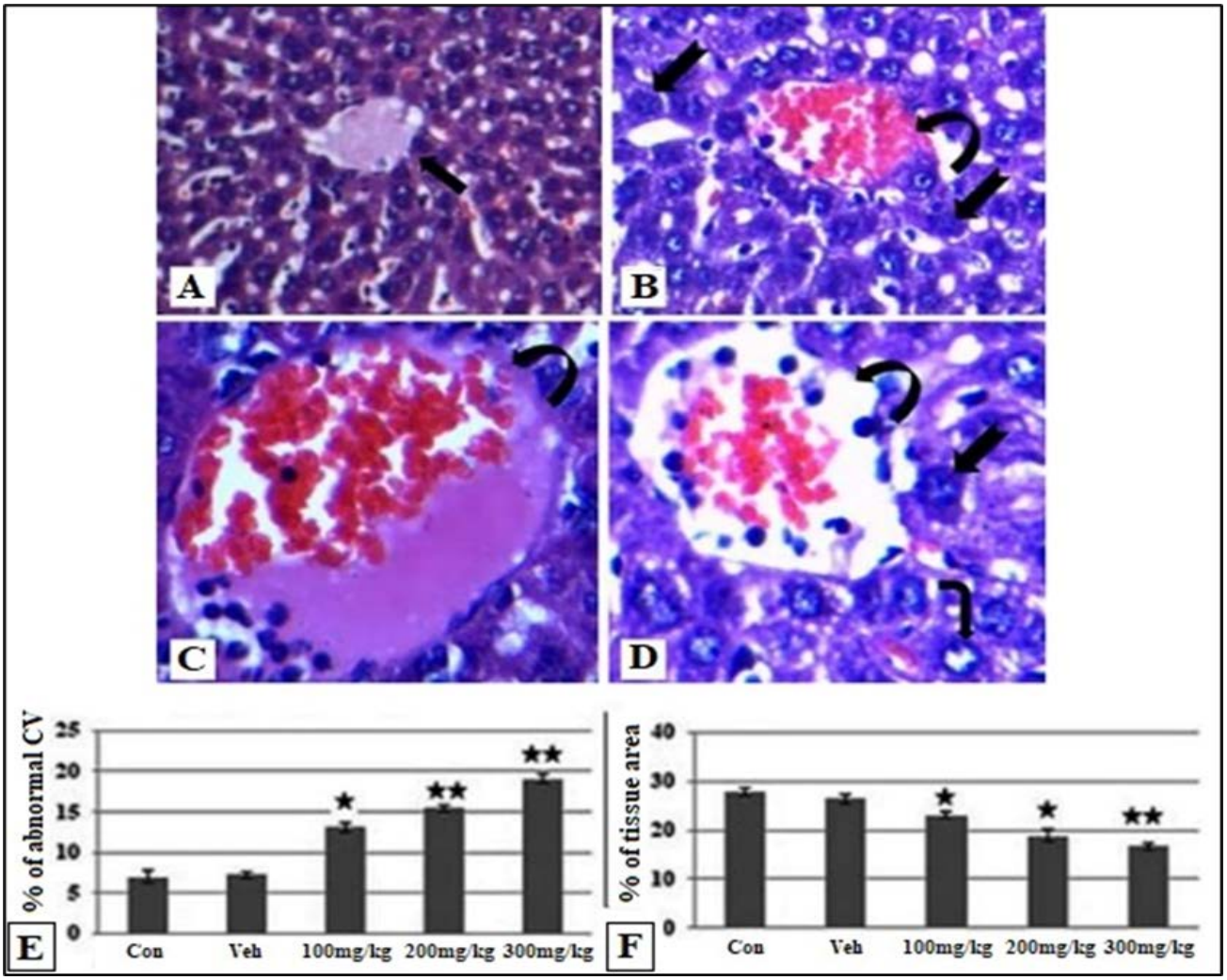


Figure 5: A – D: Photomicrograph of Haematoxylin and eosin-stained histological structure of liver in control and BNE treated solid tumour bearing mice. A: Section of liver in control mice. Straight arrow indicates normal CV or central vein (10 x 10 magnifications). B: Section of liver in BNE treated mice. Curved arrow indicates highly ruptured and congested CV; notched arrows indicate normal hepatocyte (10 x 20 magnifications). C. Section of liver in BNE treated mice; Curved arrow indicates highly ruptured and congested, large CV (10 x 20 magnifications). D. Section of liver in BNE treated mice. Curved arrow indicates highly ruptured and congested CV; notched arrow indicates normal hepatocyte and bent arrow indicates vacuolated hepatocyte (10 x 40 magnifications). E. Graphical representation revealed that the percentage of abnormal CV was significantly very high (**p>0.001) in 200mg BNE/kg and 300 mg BNE / kg b. w. treated series. F. Graphical representation revealed that the percentage of tissue area in 300 mg BNE / kg b. w. treated mouse was significantly (**p>0.001) very low in comparison to control and other treated mice. Values are expressed as mean ± SE (n=3).

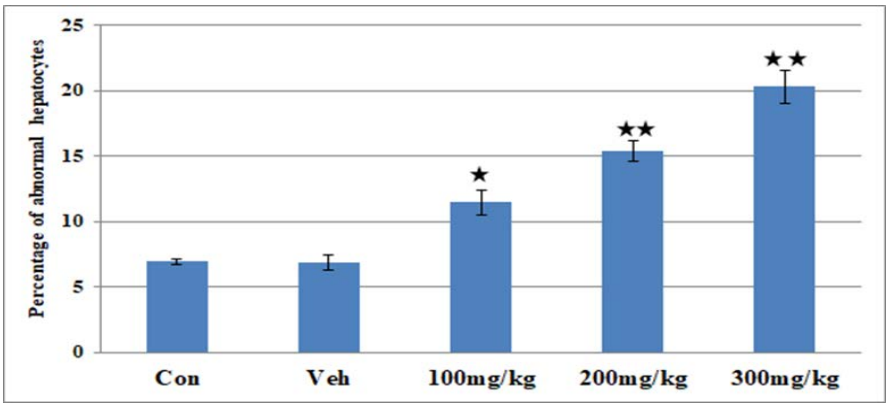


Figure 6: Graphical representation revealed that the percentage of abnormal hepatocytes in 300mg BNE / kg b. w. treated mouse was significantly more (**p>0.001) in comparison to control, vehicle and other treated groups. Values are expressed as mean ± SE (n=3).

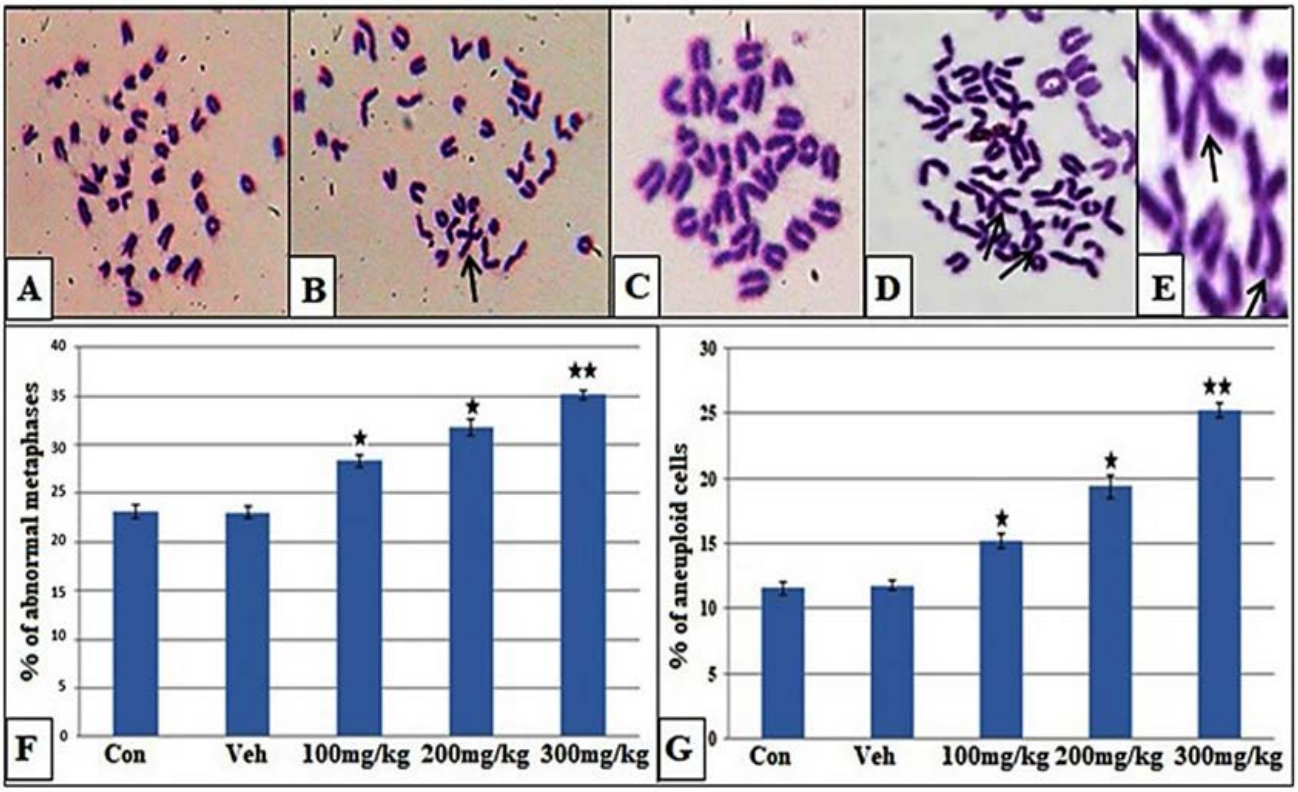
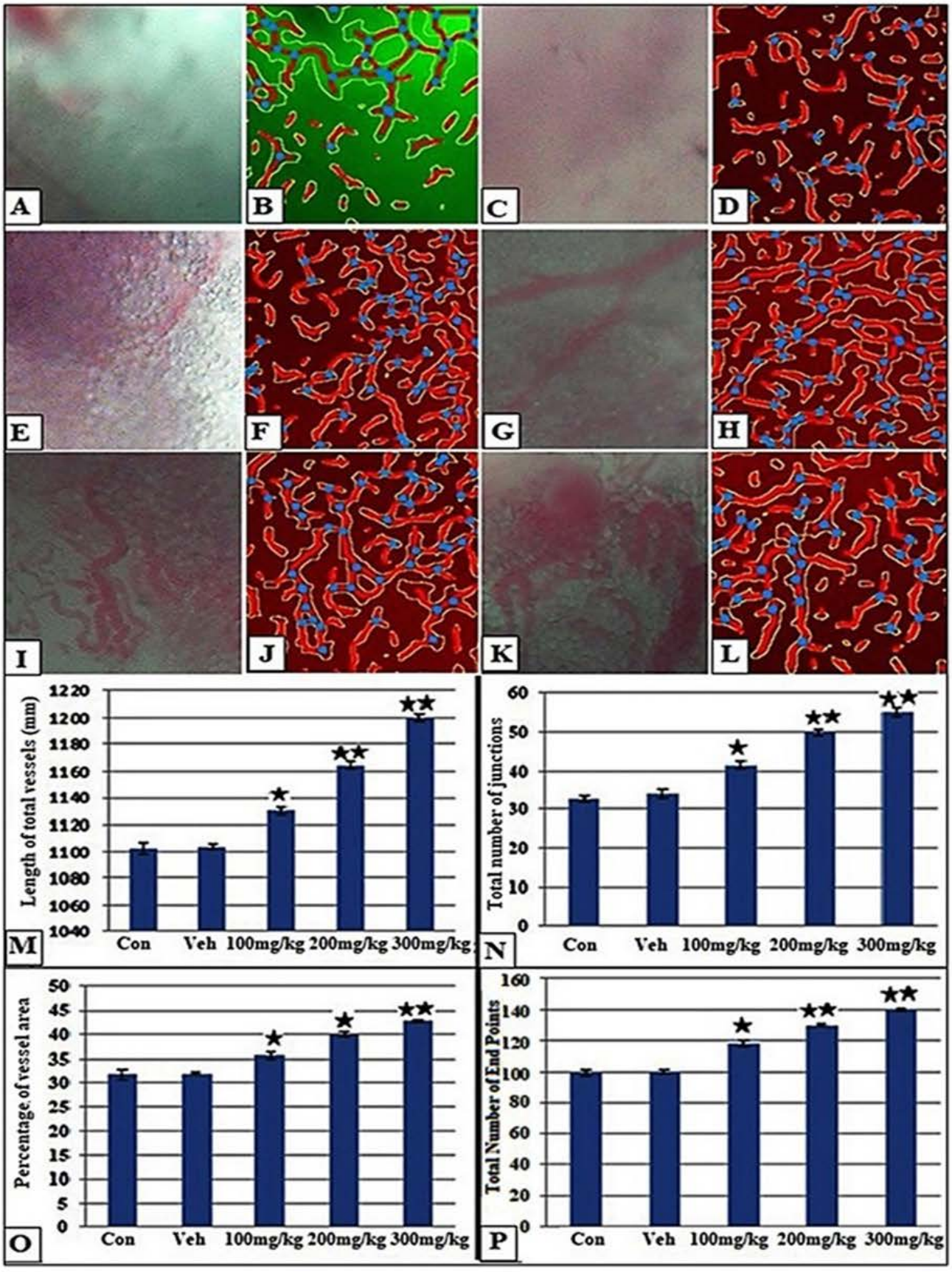


Figure 7: A- E: Somatic metaphase prepared from bone marrow of control and treated mice (10 x 100 magnifications). A. Somatic metaphase of control tumour bearing mouse without any aberration. B. Metaphase cell with one metacentric chromosome (arrowed) in treated series. C. Aneuploid (hypodiploid condition) metaphase cell. D. Metaphase cell with multiple aberrations (arrowed) i. e. two metacentric chromosomes E. Enlarged view of metaphase cell showing two metacentric chromosomes (arrowed). F. Graphical representation revealed that abnormal or affected metaphase cells in different treated mice were gradually increased. But the percentage of abnormal metaphase cells was significantly (**p>0.001) more in 300mgBNE /kg b. w. treated mouse. G. Graphical representation revealed that the percentage of aneuploid cell was significantly (**p>0.001) more in 300 mg BNE / kg b. w. treated mouse in comparison to control and other treated mice. Values are expressed as mean ± SE (n=3).

Figure 8: Effect of betel nut extract on vascularisation in the solid tumour bearing mouse. A= control (without BNE) B= Control output, C=Vehicle (distilled water injected tumour bearing mouse) D=Vehicle output, E=100mg BNE /kg body weight (b. w), F=100mg BNE/kg b. w. output, G=200mg BNE/kg b. w. H= 200mg BNE /kg b. w. output, I= 300 mg BNE /kg b. w. J= 300mg BNE/kg b. w. output, K=300mg BNE /kg b. w. L=300mg BNE /kg b. w. output, M. Graphical representation revealed that the length of total vessels increased in treated groups. In 100 mg BNE treated group the length of blood vessels increased significantly (*p>0.05), but it was highly significant (**p>0.001) in 200mg and 300mg treated mice. N. Graphical representation revealed that the total number of blood vessel junctions increased in treated groups. In 100 mg BNE treated group the blood vessel junctions increased significantly (*p>0.05), but it was highly significant (**p>0.001) in 200mg and 300mg BNE treated series. O. Graphical representation revealed that the percentage of vessel area increased in treated groups. In 100 mg BNE and 200 mg BNE treated groups the percentage of vessel area increased significantly (*p>0.05), but it was highly significant (**p>0.001) in the 300mg BNE treated series. P. Graphical representation revealed that the total number of vessel end points increased in treated groups. In 100 mg BNE treated groups the vessel end points increased significantly (*p>0.05) whereas it was highly significant (**p>0.001) in the 200mg BNE and 300mg BNE treated series. Values are expressed as mean ± SE (n=3).



CONCLUSION

Several analyses such as haematological, histopathological in respect to hepatotoxicity, cytological toxicity and angiogenesis were done to understand the basis of tumourigenic property of betel nut. Our present study suggests that betel nut possesses potent carcinogenic

activities and enhances solid tumour growth with toxic mutagnic potentially (Fig .9)

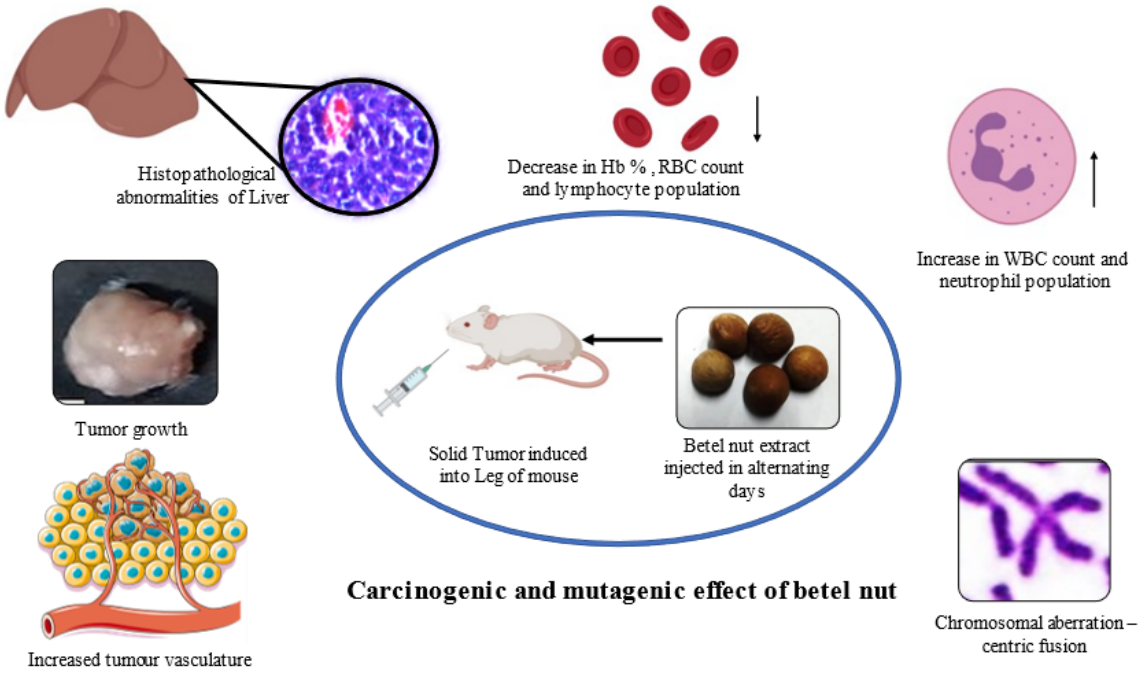


Figure 9: Carcinogenic potentiality induces a solid tumour growth with haematological toxicity, hepatotoxicity, chromosomal abnormality and increased tumour angiogenesis

ACKNOWLEDGEMENT

The research work is dedicated to late Professor Samar Chakrabarti, Cancer Cytogenetics Unit, Department of Zoology, Burdwan University, Burdwan, India. Authors are thankful to Dr. R.N. Boral and Dr. C.K. Panda, CNCI, for technical help. Authors are grateful to Ms Srijita Banerjee, German Cancer Research Center (DKFZ), Heidelberg, Germany for help. Authors are also grateful to Dr.Sukanti Dutta, Associate Professor, Department of English, City College Commerce and Business Administration, Kolkata - 700012 for critical review of the manuscript. Authors are grateful to Dr. Saswati Sanyal, Principal, Rammohan College, Kolkata for support and encouragement. The work gained support from the faculties of the Department of Zoology, Rammohan College, Kolkata. University Grants Commission is also acknowledged for granting research instrument [University Grants Commission, XI Plan Additional Grant, 1st Phase, Reference no AGW- 287/11-12 (ERO) dated 12.07.2012.]

CONFLICT OF INTEREST: None

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