Original Research Article

Differential Uterine Protein Expression Induced by Crude Bark Extract of *Dysoxylum alliarium* During Day4-8 of Gestation is Associated with Increased Serum Profile of SGOT & SGPT but not Cellular Toxicity in Rat

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Abstract: Plants and herbs have formed the basis of various traditional medicine systems and folk medicines that have been practised for thousands of years during the course of human history. *Dysoxylum alliarium* has been found to be used by the Adi tribe of Arunachal Pradesh as traditional fertility control drug for domesticated animals. Present study aims at investigating the effects of crude bark extract of *Dysoxylum alliarium* on the uterine proteins during early gestation in rat (D4 – D8) and hepatic toxicity if any. Methanolic crude bark extract (CBE) was administered to pregnant rats from D1 to D7 of gestation. Administration was done through oral route in a dose of 500mg/Kg body weight every morning in between 7.00 – 9.00 hrs. Samples were collected from D4 to D8 in five different groups. The separation of uterine proteins was performed by SDS-PAGE and the serum profile of SGOT and SGPT was analysed. Histological study of liver tissue was done by routine H&E staining to determine the cellular toxicity of the crude bark extract of the plant. The results showed that the CBE stimulates the synthesis of high molecular weight protein on Day 4, 7 and 8 of gestation. The compound(s) responsible for the change of cellular protein profile need to be investigated. Increased level of SGOT and SGPT was reported after the treatment of the CBE. Infiltration of neutrophils in the liver is seen suggesting cellular necrosis, but without evidence. Further study on these aspects may lead to the development of an effective fertility control drug.

Keywords: Anti implantation, Crude bark extract, *Dysoxylum alliarium*, Fertility control, Traditional medicine

Introduction

Use of plant resources for reproduction regulation is an age old practice of communities in different parts of the globe. A good number of modern drugs have been isolated from natural sources (Newman *et al.*, 2003) and they are expecting for more in coming years. With other branches of healthcare, traditional medicines are regularly explored for development of a effective reproductive health friendly contraceptive/ abortifacient drug. *Dysoxylum alliarium* is considered as an important medicinal plant used by 'Adi tribe', a tribal group of people of North East India. Collection of first hand information reveals that this plant is being traditionally used by the indigenous people of these regions for fertility control especially of domesticated animals e.g. dogs and pigs. The dry bark powder is orally given in mixture with other food stuff to domestic pets immediately in post coital period to avoid unwanted pregnancies.

The plant *Dysoxylum alliarium* belonging to the family Meliaceae is mainly distributed in parts of Arunachal Pradesh, Assam, Sikkim, Meghalaya and other north eastern part of India (Hajra *et al.*,1997). The plant is an evergreen

tree, bark splits from below and thus envelope like scales; dark brown in colour. Flowering and fruiting season is from June to August. It has been hypothesized that, the herbal component(s) of the bark of this tree with abortifacient/ antifertility property mediate its effect by various ways in the targeted animals' reproductive organ. Therefore, the present investigation was undertaken to study the effect of the crude bark extract (CBE) of *Dysoxylum alliarium* as an abortifacient agent disturbing the early gestational uterine environment targeting the endometrial proteins.

Materials and Methods

Collection and Preparation of plant extract

The bark of *Dysoxylum alliarium* was collected, cleaned and shade dried. The dried bark was chopped into small pieces then ground to make 60 mesh powder. The powder was soaked in methanol for a period of 72 hours at room temperature (25±2°C) and subsequently filtered. The filtered solution was allowed to dry in room temperature. The semi solid methanolic extract was administered to the experimental animal.

Experimental animal and experiment design

Adult cyclic female albino rats (150 ±10 g body weight) were used in the present investigation. Animals were kept in the central animal facility of Rajiv Gandhi University under uniform husbandry conditions and natural light and temperature. Rats were fed with routine diet (Bengal gram, corn) and water *ad libitum*. The threshold dose of crude bark extract was determined earlier in the present laboratory (Moushumi Das *et al.*, 2013). The rats were treated with crude bark extract through oral route at a dose of 500mg/kg body weight/day during the periimplantation period (Day 1 to Day 8 of gestation). To determine the pregnancy, vaginal smear of normal cyclic female albino rats were examined daily. The female found in the proestrus phase were allowed to mate with male of proven fertility in the ratio of 1:2 (1 male : 2 females). Positive mating was verified with the presence of thick clumps of spermatozoa in the smear. The rats showing spermatozoa in the smear were separated and the day was considered as the day 1 of pregnancy. The whole experiment was performed in five groups. In Group I, rats were treated with CBE from Day 1-Day 4 of gestation. In Group II, rats were treated with CBE from Day 1-Day 5 of gestation. In Group III, treatment was given from Day 1-Day 6 of gestation, Group IV: Day1-Day7, and Group V: Day1-Day8 of gestation respectively. The rats were sacrificed on Day 4, Day 5, Day 6, Day 7 and Day 8 in each group respectively during 18.00 to 19.00 hrs.

Each day from day 4 to day 8 of gestation one group of CBE treated (n=6) and one group of control females (n=6) were sacrificed for sample collection. Uterine horns were collected to study the uterine protein profile for both control and treated females. Prior to sacrifice of the animals, blood samples were collected by puncturing the heart by 21 G BD needle under anaesthesia. Serum samples were kept in -20^{0}C to study the level of hepatic enzyme SGOT (AST) and SGPT (ALT). One group of rats were also treated with CBE from Day 1 to Day 15 of pregnancy to study the long term toxicological effect of the CBE on hepatic tissues.

Study of uterine protein by SDS-PAGE

The uterine protein samples were prepared and studied by discontinuous single dimensional SDS-PAGE following the method of Ausubel *et al.*, (2002). The uterine horns were collected and placed in the sample buffer. Following homogenisation and 10 minutes boiling and instant cooling the samples were centrifuged at 10,000 rpm for 10 minutes. The supernatants were collected and stored in -20^{0}C until use. Quantitative estimation of the proteins was done following the method of Bradford (1976). SDS-Polyacrylamide gels were prepared using standard protocol. Sample containing 80-100µg protein mixed with glycerol was loaded in each lane of the gel. The proteins were stained with commassie brilliant blue dye. The bands in the gel were studied in a gel doc system (Geliance 200 imaging system). The molecular weights of the separated uterine proteins were detected by comparing with a standard molecular weight marker (Novagene, Protein marker 10-225 kDa cat No. # 69079-3).

Determination of Serum glutamic oxaloacetic transaminase (SGOT) & Serum glutamic pyruvic transaminase (SGPT) level in serum

The level of SGOT also known as aspartate aminotransferase (AST) and SGPT known as Alanine aminotransferase (ALT) was determined in the serum sample as the toxicological marker enzyme to study the toxicological effect of the CBE. Blood samples were collected from the females on day 4 to day 8 of gestation as well as day 15 of gestation (females treated for long term: day 1-day 15 of gestation). Serum samples were separated by centrifugation at 3000 rpm for 8 minutes. The SGPT and SGOT levels were analysed using the kits from Crest Biosystems following the methods of Reitman and Frankles (1957).

Histological study of Liver

Liver tissues collected from treated females (day 1-15 of gestation) were processed for histological study following the method of Culling (1972). Serial sections (6 - 7μm) were stained with Delafield hematoxylin and eosin. Alternate sections were observed and appropriate areas were photomicrographed to collate the most significant results in histoarchitecture of hepatic tissue.

Statistical analysis

The data of serum level of enzymes (SGOT and SGPT) were statistically analysed using students t-test. A difference was considered significant if the double tailed (paired) probability value $p < 0.05$.

Results

Day 4 of gestation: The proteins separated by SDS-PAGE on day 4 of pregnancy in both control and *Dysoxylum alliarum* (CBE) treated rats have been presented in Fig.1.a. The control females on day 4 of pregnancy showed all together ten protein bands with mol. Wt. Range 6.5 kDa to 205 kDa (band A – J). The protein band having molecular weight 66 kDa (band E) has been found to be expressed in the highest intensity, while the protein band with highest molecular weight (205 kDa, band A) expressed with lowest intensity in the control (day 4) rat uterus.

However, following the administration of the CBE from day 1 to day 4 of gestation, two high molecular weight protein bands (band B & C) in between 205 kDa and 96 kDa expressed in higher intensity than that of the control uterine protein bands. While five protein bands (band F, G, H, I & J) having molecular weight in between 66 kDa and 6.5 kDa have been found to be down regulated following CBE administration.

Day 5 of gestation: The protein expression pattern in the uterus on day 5 of gestation (Fig.1.b) showed 9 distinguishable protein bands within the molecular weight 6.5 kDa to 205 kDa. Two protein bands within the range of 45 kDa (band F) and 66 kDa (band G) have been found to be intensely expressed in both control and CBE treated females. The protein band C of approximately 125 kDa, has been stimulated to express in the CBE treated rats' uteri but was not observed in the control day 5 rats. Expression of high molecular weight protein bands in between 66 kDa to 205 kDa was more intense in CBE treated females than that of the controls.

Day 6 of gestation: On day 6 of gestation (Fig.1.c) both control and treated females' uteri exhibited intense expression of two protein bands having molecular weight >45 kDa (band H) and 66 kDa (band G). Two protein bands (Band I & J) of molecular weight approximately 43 kDa and 6.5 kDa have been appeared diffused indicating doublets (multiple numbers of proteins together).

Administration of CBE induced expression of a high molecular weight protein (> 205 kDa, band A). The protein band C showed a doublet pattern while, the low molecular weight protein band (mol wt. 29 kDa; band J) expressed in lower intensity in comparison to that of the control females.

Day 7 of gestation: The day 7 pregnant control and treated female rats (Fig.1.d) uteri showed expression of highest number of protein bands. The protein bands A (205 kDa), B (<205 kDa), E (>66 kDa), F (>66 kDa) and G were found to be up regulated following the administration of CBE.

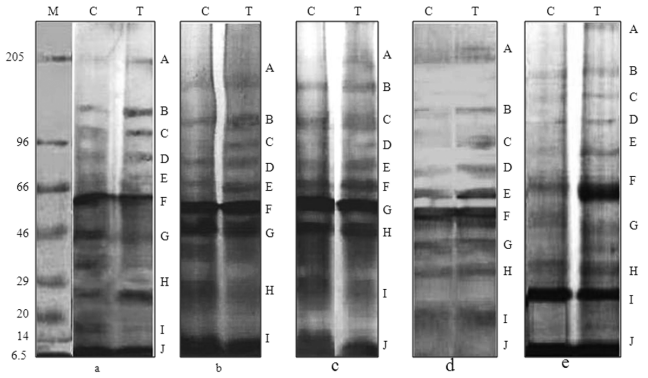

Fig.1. Uterine protein profile separated by SDS-PAGE of control (C) and CBE treated (T) rat uterus on day 4(a), 5(b), 6(c), 7(d) and day 8(e) of gestation. Molecular weight of the proteins are presented in kilodalton (kDa) and compared with standard molecular weight marker (MW). M=Molecular weight marker, C=Control, T=Treated

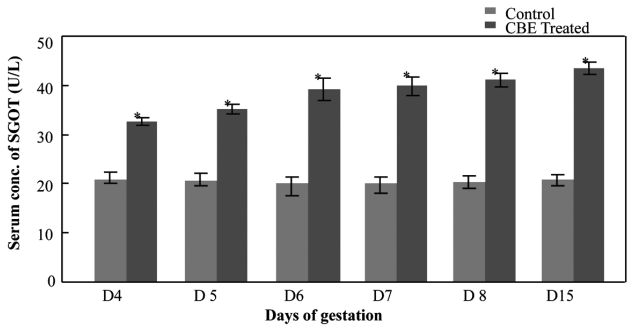

Fig.2.a. SGOT(AST) profile of control and CBE treated rats during the periimplantation period (day 1 to day 8 of gestation) and following a long term treatment (day 1-15 of gestation) of CBE. Values are mean ± S.E. ($P < 0.05$)

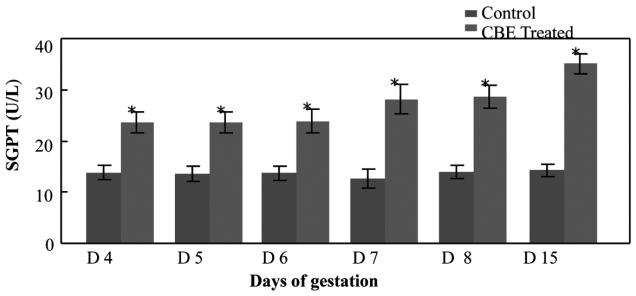

Fig. 2.b. SGPT (ALT) profile of control and CBE treated rats during the periimplantation period (day 4 to day 8 of gestation) and following a long term treatment (day 1-15 of gestation) of CBE. Values are mean ± S.E. ($P < 0.05$)

Day 8 of gestation: A total of ten protein bands were expressed in the (Fig. 1.e) uterine tissues on Day 8 of gestation. Following CBE administration, expression of new protein bands of molecular weight within the range of 96 kDa (band E) to 29 kDa (band G) was found. As noted in the study, the protein band of > 25kDa (band I) molecular weight could be regarded as day 8 gestation specific, as this protein band was expressed both in control and CBE treated females in similar intensity. The other protein bands, (band A, B, C, F) were expressed comparatively in higher intensity in CBE administered females.

Serum profile of SGOT and SGPT

Serum glutamic oxaloacetic transaminase: The result of the present investigation showed that the (Table 1) oral administration of CBE significantly ($P<0.005$) increased the level of the enzyme (SGOT) in serum of pregnant rats in comparison to control (control level ranges between 20U/ml to 21U/ml). Elevation of the enzyme level to a value of 32.66 ± 0.83 U/ml on day 4 further increased to 35.17 ± 0.92, 39.25 ± 2.29 U/ml, 39.83 ± 1.95 U/ml and 41.16 ± 1.4 U/ml respectively on day 5, 6, 7 & 8 of gestation (Fig. 2.a). Treatment with CBE from day1-15 of gestation showed the highest increase of the enzyme level (43.5 ± 1.29) U/ml against the normal control value of 20.91 ± 0.97U/ml.

Serum glutamic pyruvic transaminase: The control females showed approximately similar level of SGPT (U14.3 ±1.97 U/ml to 14.3±1.97 U/ml) from day 4 to day 8 and on day15 of gestation. The CBE treated females on their gestation day 4, 5 and 6 showed significantly ($P<0.005$) elevated levels of the enzyme with the values of 23.66 ± 2.12U/ml, 23.59 ± 2.08U/ml and 23.83 ± 2.34 U/ml respectively against the respective control values of 13.85 ±1.33U/ml, 13.55 ±1.44U/ml and 13.72 ±1.38U/ml. However, no marked differences were observed in the range of enzyme levels from treated day 4 to day 6 of gestation (Fig. 2.b). The level of SGPT increased more than two folds than that of the control level from day 7 of gestation onward and exhibited a highest increase in value i.e. 35.08 ± 1.97U/ml in Day 1-Day 15 treated rats (Table1).

Histological studies of liver

The liver of the control females showed distinct polyhedral structure of hepatic lobule with the radially arranged hepatocytes. The hepatocytes of control females appeared

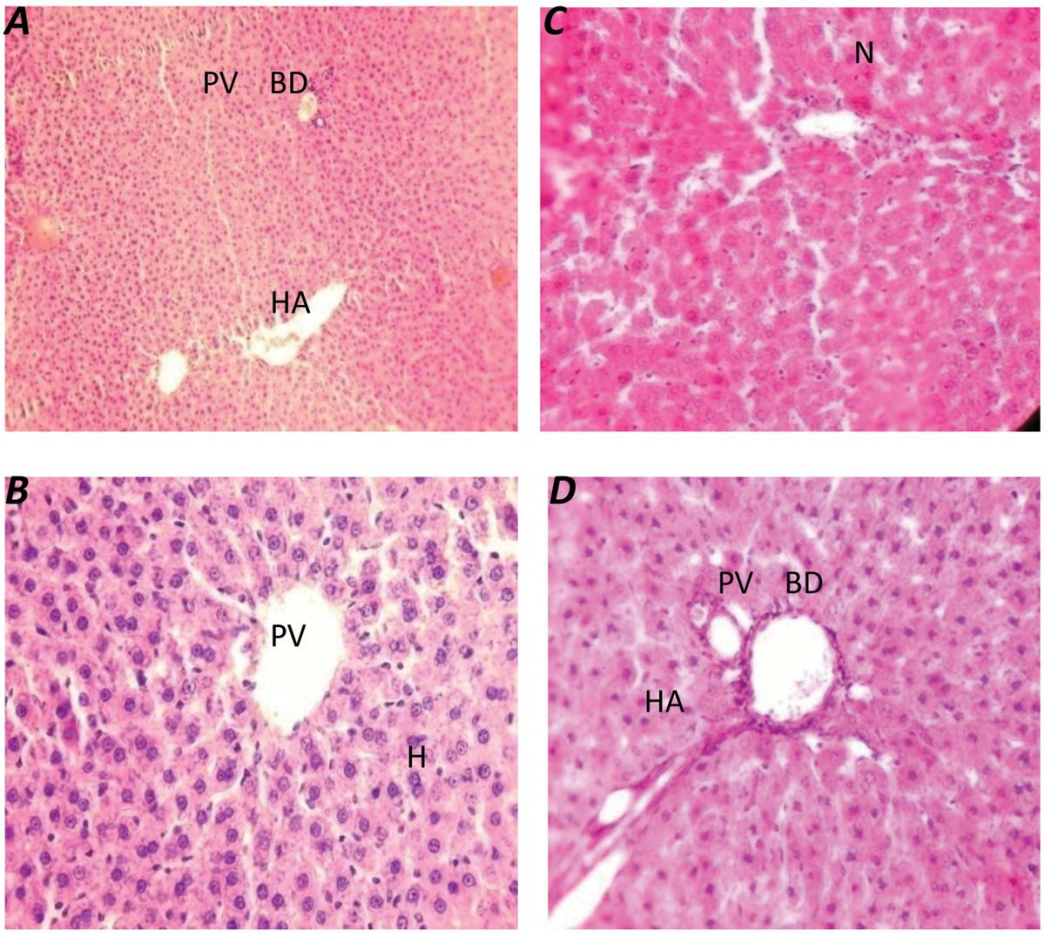

Fig.3. The histological structures of liver of control and CBE treated females (Day 1-15 of gestation). The control females liver (A 10x, B 40 x) showed compact hepatic lobule with portal vein (PV) and the bile duct (BD), branches of hepatic artery (HA), the hepatocytes (H) and the sinusoids (S). Administration of CBE (C 40x & D 40x) results in infiltration of neutrophils (N) surrounding the bile duct.

with distinct nucleus with clear cytoplasm indicating functional liver tissues.

Following the administration of CBE consecutively for 15 days from day 1 of gestation, the cells of the hepatic cords showed a slightly higher order of basophilia (Fig. 3.C) with induced infiltration of neutrophils surrounding the bile ducts and the sinusoids indicating initial stages of cellular necrosis. Cellular necrosis of the hepatocytes as expected from increased level of AST and ALT in serum was not observed in the long term CBE treated liver.

Discussion

A number of hormones, growth factors, and cytokines are involved in the process of uterine remodelling during early pregnancy and embryonic development. Multiple numbers of genes are expressed producing structural and functional proteins for cellular remodelling and embryo implantation. In this stage, disappearance of many inhibitory proteins and /or emergence of activation proteins have been suggested earlier (Surani, 1975; Lejeune *et al.,* 1985). The appearance of a 43kDa (MW) protein has been considered as one of the most marked changes at the time of blastocyst invasion of the uterine epithelium (Jacobs *et al.*, 1987). Generally, during pregnancy despite of large increase (13-fold) in uterine size, the fractional rate of protein synthesis (measured in vivo) remained unchanged when compared with non pregnant uterus (Morton *et al.,* 1986).

Table 1. Studies of SGOT (Aspartate aminotransferase) and SGPT (alanine aminotransferase) profiles in control and CBE treated females during gestation. Values are expressed as mean ± SE.

Parameter	Group	Day 4	Day 5	Day 6	Day 7	Day 8	Day 15** (long term CBE treatment)
SGOT	Control	20.83±1.45	20.5±1.52	20±1.29	20±1.29	20.41±1.12	20.91±0.97
(AST)	(N = 6)						
(U/L)	Treated	32.6±0.83*	35.1±0.92*	39.2±2.29*	39.8±1.95*	41.16±1.4*	43.5±1.29*
	(N = 6)						
	Control	13.85±1.33	13.5±1.44	13.7±1.38	12.6±1.82	13.96±1.37	14.3±1.97
SGPT	(N = 6)						
(ALT)	Treated	23.66±2.12*	23.59±2.08*	23.83±2.34*	28.16±2.84*	28.6±2.23*	35.0±1.97*
(U/L)	(N = 6)						

N = Number of animals in each group. C= Control, T= CBE Treated. $p < 0.05$

** Number of animal C=3 & T=4 during D1-15 CBE treatment.

In the present study, the CBE has been found to stimulate synthesis of a high molecular weight protein (Fig.1) on day 4, 7 and day 8 of gestation. The functional role of this protein in uterus during uterine maturation phase is not known. The other proteins expressed with altered intensity in the treated rats uteri may be attributed to the estrogenic compound present in CBE. Many protein molecules are inevitable for establishment of pregnancy. Modulation of these protein molecules quantitatively or qualitatively may induce deleterious effects on feto-maternal unit. The requirement of expression of protein may vary on different days of periimplantation period depending upon the stages of embryonic development and subsequent changes of the maternal tissues to support the embryonic growth. The evidence came from the present observation that protein molecule of approximately 66kDa has been intensely expressed on day 5 (band F, Fig.1.b) and day 6 (band G, Fig. 1.c) in both control and CBE treated females. In contrast, this protein has been expressed in lower intensity on day 7 and 8 of gestation in control females' uteri indicating decreased physiological role on these days of gestation. The differential expression and requirement of proteins for gestational support has been more pronounced on day 8 of gestation as one protein molecule within the range of 20 to 29 kDa appeared in higher intensity in both control and CBE treated females' uteri and another molecule of >66 kDa appeared intensely in CBE treated uteri. As mentioned above, the synthesis and appearance of higher molecular weight protein is reported to be increased during early pregnancy (Surani, 1976). Synthesis of many proteins is up regulated during the period between day 5^{th} and day 6^{th} of pregnancy in rat (O'Grady and Bell, 1977). The role of estrogen in inducing uterine protein synthesis during estrous cycle and gestation has been discovered earlier (Katzenellenbogen and Greger, 1974; Walker *et al.*, 1976; Iacobelli *et al.*, 1977). Previous experiments showed that estradiol induces synthesis of nucleic acid and protein in the uterus and blastocyst (Mohla *et al.,* 1970). Notides and Gorski (1966) first reported the induction of new protein (induced protein) in the uterus of rats and mice on administration of estradiol-17β. Furthermore, Hazarika *et al.,* (2002) reported the expression of two high molecular weight proteins in between 55 kDa and 68 kDa in the uterus of OVX female in response to exogenous estradiol-17β (subcutaneous injection). However, functionally the increased expression of protein and synthesis of new protein may exert an adverse effect in the process of implantation and the maintenance of pregnancy.

Continuous exposure of the liver tissues to the toxicants may result in hepatocyte injury which may cause chronic hepatitis and fibrosis ultimately leading to the life threatening complications of portal hypertension and liver failure. The 'knodell histology activity index' has been widely regarded as the benchmark for reproducible description of the various morphological lesions of chronic hepatitis (Knodell *et al.*, 1981). In recent years, the METAVIR scale has been widely used to measure the degree of fibrosis on hematoxylin-eosin stained sections (Pik-Yuen Cheung *et al.,* 2006; Bedossa

and Poynard, 1996). In the present investigation, infiltration of neutrophils in the liver following long term CBE treatment led to hepatocyte necrosis resulting in increased level of SGOT and SGPT. A higher ordered hepatic damage (F3 and grade3) has not been observed following CBE treatment for consecutive 15 days. The toxic effects of CBE on rat hepatic tissues as described above may be induced by the various agents present in the bark of the plant *Dysoxylum alliarium*. The thin layer chromatographic fraction of the plant *Dysoxylum alliarium* suggested the presence of phytoestrogens in the bark of the plant (Das *et al*., 2013). In addition to the phytoestrogens present in the CBE, presence of other toxicants and heavy metals cannot be ruled out. The hepatic necrosis might be due to the lipid peroxidation supposed to be induced by the plant product. Aqueous extract of *Eucalyptus globules* is known to be stimulating the membrane lipid peroxidation with a significant increase in the malondialdehyde (MDA), a major product of lipid peroxidation (Arise *et al*., 2009). Solomon *et al.* (1993) reported the toxic effect of the crude root extract of *Plumbago rosea* on rats following a treatment of 30 days at a dose of 50mg/kg body weight. A detailed study of toxic effects of potential active component present in the bark of *Dysoxylum alliarium* shall be required to develop a health friendly 'lead' from this plant. Isolation of the compound(s) and chemical characterization of this plant derive compound(s) shall be the future direction of research on this potential herbal product for development of an alternative health friendly abortifacient medicine.

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