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A N I N T E R N A T I O N A L J O U R N A L

Implications arising from the use of *Cymbopogen proximus*; proximal on placenta of pregnant Albino rats

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ABSTRACT

Halfa-bar (Cymbopogon proximus), is an aromatic grass widely growing in Upper Egypt. This herb is recommended for medical purposes as an effective diuretic, renal or abdominal antispasmodic agent. Objectives of this study: Evaluate the potential effects of Halfa-bar on the pregnant albino rats during the gestation period. Material and methods: The virgin female rats mated with male then the pregnant rats treated orally with Human Equivalent Dose (HED) of the proximol which equivalent 0.05 mg/ kg rat from 5th -18th Gestational Day (GD). At day 20 of pregnancy, all rats were anesthetized and killed to obtained maternal –fetal data (placenta). Results: The current study indicated that, there is statistically significant ($P \leq 0.05$) reduction in the treated placental weight. Also, the light microscopic examination of the placental specimens using haematoxylin and eosin (H&E) staining revealed the presence of various vacuoles in the cytoplasm and nuclei of the giant cells. There is an increase in the number of apoptotic cells and irregular dilatation of maternal sinusoids in the labyrinth zone. Else, microscopic investigation showed a depletion of the glycogen content in the basal and labyrinth layers and a positive caspase-3 in the spongitrophoblast cells. In the treated group, reduction in level of catalase activity (CAT) and significant elevation ($P \leq 0.05$) in the level of malondialdehyde (MDA) were recorded. Conclusion: The pathological effects in placenta may be due to the accumulation of proximal and transplacental passage.

Key word: *Cymbopogen proximus*, placenta, *Rattus norvegicus*

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INTRODUCTION

The relationships between the mother and fetus are very complex; these relations occur through the placental tissue that control development of the fetus. So, the placenta is the principle link between mother and its fetus, being responsible for the transfer of nutrients from maternal to fetal blood and for the clearance of waste metabolites from fetal blood across the labyrinth layers of the placental barrier. In addition, the placenta is a target organ for drug- or chemical-induced adverse effects. Drug- or chemical induced placental functional depression and injury subsequently result in abnormal fetal growth or development leading to delay in growth of fetuses, fetal resorption and malformation. It is worthy to mention that; placenta is thus determinant for pregnancy success.

Halfa-bar (*Cymbopogon proximus*), is an aromatic grass widely growing in Upper Egypt, its genus *Cymbopogon*, belong to family Gramineae. This herb is recommended for medical purposes as an effective diuretic, renal or abdominal antispasmodic agent. Halfa-bar acted through relaxation of the smooth muscle fibers without abolishing the propulsive movement of the tissue, thus, it is usually used in the expulsion of renal and ureteric calculi [1]. Terpenes, tannins, flavonoids, saponins, alkaloids, carbohydrate or glycosides, and phenolic glycosides are the phytochemical compositions which are found in the aqueous extract of *C. proximus* [2], [3].

Even for efficient and documented herbal medicinal products, the toxicity can be relatively undetected; unlike, the conventional drugs research and development, the toxicity of usual herbal medicines is not often estimated [4], [5], [6]. The majority of the population however does not give awareness, believing that, if these products have been used yet, they should be free from toxicity [7], [8a, b], [9], [10], [11].

Numerous mechanisms differ among various cell types are contributed in the link between the damaging effects on preganant rats and their developing fetus. The presence of these mechanisms may act simultaneously or consecutively as a particular challenge to investigators. There are numerous potential mechanisms through which *Cymbopogon proximus* act on the fetus, many of which result in cell death by necrosis and/or apoptosis. Among these mechanisms are oxidative stress elevation and mitochondrial damage [12].

Generally in developing countries, the use of medicinal plant products to treat different diseases is a common practice. However, a lack of information on the adverse effects of these plants raises questions on their safety and possible adverse side effects. Halfa-bar may affect directly, by acting on fetal tissue, and indirectly, by interfering with the maternal support of the growing fetus. Such indirect mechanisms include altering the placenta's ability to give the essential nutrients to the developing fetus. So, this study was undertaken to evaluate the potential implication of *Cymbopogon proximus* using placenta tissue of pregnant rat as an animal model.

MATERIALS AND METHODS

Drug Used

Halphabarol, the active principle of PROXIMOL is the most potent out of four antispasmodics present in the national desert weed "*Cymbopogen proximus*" or "Halfa Bar". Tablets of Proximol were purchased from Kahira pharmathetical company, Cairo, Egypt. The concentration of active ingredient in each tablet is 0.4 mg Halphabarol. Proximol was administrated orally using oral stomach tube at a dose of 0.05
mg/kg rat corresponding to the human therapeutic dose level.

Animals

Vigin female and male albino rats, *Rattus norvegicus*, weighing about 170-180g were used in the present investigation. The animals were obtained from the animal house of the Faculty of Veterinary Medicine, Giza, Egypt. Before starting the experiment, animals were allowed to acclimatize for at least 7 days. Pregnancy was established by housing females in the pro-estrous and oestrous stages with healthy fertile males over night; 12-h light/dark cycle (2:1) under controlled environmental condition of temperature (25±2^{o}c), humidity (60±20%) and given feed and water ad libitum. The next morning, females with positive vaginal plug or vaginal smears were considered pregnant, and the day of detection was defined as the first day of pregnancy or gestation [13].

Experimental protocols and procedures used in this study were approved by the
Cairo University, Faculty of Science, and Institutional Animal Care and Use Committee (IACUC) (Egypt) (CUFS/F/46/14).

Experimental studies
Pregnant rats were allocated into two groups (15 per group). The control group received saline water and the experimental groups received 0.05 mg/kg of proximol diluted in saline. The dosing regimen was based on human equivalent/therapeutic dose (HED). The drug were administered orally by gavage from day 5 up to 18 of pregnancy, defined as the critical period for the structural development span of the embryonic stage for rats [14]. In the twentieth day of gestation, all animals were euthanized with sodium pentobarbital. Cesarean sections were performed on the pregnant rats and the gravid uteri were removed. The fetus and its corresponding placenta were extracted from each uterus). The embryonic membranes around the fetuses were removed and placentas were separated from the fetuses. The following investigations were carried on the collected placentas.

Histopathological studies
Maternal placentas of the treated and control groups were firstly fixed for histological investigation by light microscopy using 10% formalin solution for 24 hrs. Washing was done in running water to remove excess fixative then dehydration using serial ascending series of ethyl alcohol. Specimens were cleared in xylene and embedded in paraffin wax at 56 degree in hot air oven. Paraffin bees wax tissue blocks were prepared for sectioning at 5 μ thicknesses using Reichert microtome. The obtained tissue sections were mounted on glass slides, deparaffinized, stained with haematoxylin (Ehrlich) and counterstained by eosin for routine examination then examination was done through the light electric microscope [15]. For histochemical study, specimens were fixed in Carnoy's fluid. Periodic acid Schiff's reaction was used for demonstration of polysaccharides [16]. For immunohistochemistry examinations, capase-3 (apoptotic marker) positive cells were determined with streptavidinbiotin- peroxidase staining method [17].

Oxidative stress investigation
Autopsy samples were taken from the placenta of mother rats in control and treated groups and stored at -40◦C until used. Piece of each tissue was weighted and homogenized in 10 mmol/L phosphate buffer saline (PBS) as 10 % (W/V) at pH 7.4. The homogenates were centrifuged and the supernatants were taken. Estimation of catalase (CAT) was determined by calorimetric method [18] and lipid peroxidation (Malondialdehyde) (MDA) was carried out by adding reagent kits obtained from Bio Diagnostic (Egypt) to the supernatants.

Statistical analysis
All statistical analyses were performed using PASW statistical version (18). Statistical analysis was performed using one-way analysis of variance (ANOVA) to assess significant differences among the treated and control groups. P-value was used to determine significant differences. Data are expressed as means (μ) ± standard errors (SE) and $P<0.05$ was considered statistically significant.

RESULTS

Average weight of placenta
The pregnant dams treated with 0.05 mg/Kg of proximol showing reduction in the placental weight compared to control (Fig.1 & Table1). Statistically, there is significant ($P \leq 0.05$, $t= 3.084$, $df= 75.829$) reduction in placenta weight of animals that received proximol (group B).

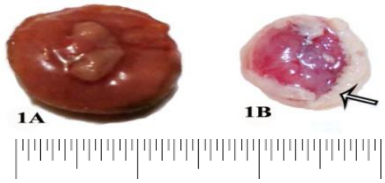

Figure 1: A Photograph of placenta of fetuses at 20th day of gestation showing decreasing in weight. A= control, B= Treated group.

Table 1: Showing effect of proximol on placenta weight at 20th day of gestation.

Group	*Placenta weight mean (g)*
Control (A)	*0.49 ±0.01*
0.05 mg/Kg (B)	*0.59±0.01*

Histopathological examination

The placenta is a transitionary tissue, forming from a maternal portion and a fetal one. Without any administration to proximol, there was no significant difference between the ratio of labyrinth area and the area occupied by spongiotrophoblasts. There is normal giant, glycogen cells and the blood vessels (Fig. 2).

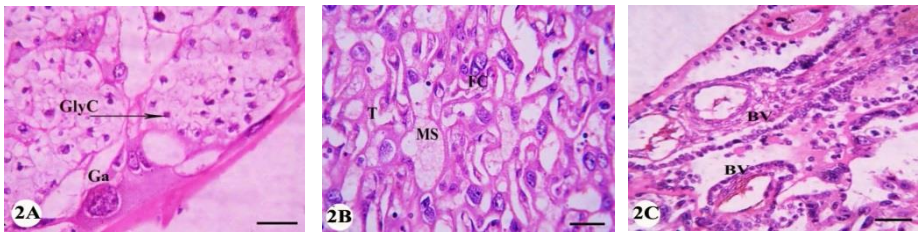

Fig. 2: A Photomicrograph of a section of placenta of control pregnant rat. H&E stain. A) Basal zone; Ga= giant cell, GlyC= glycogen cell. B) Labyrinth zone; showing trophoblastic trabeculae (T) consisting of trophoblasts and syncitiotrophoblast, fetal capillaries (FC) lined by endothelial cells containing fetal erythroblast and maternal sinusoid (MS) containing maternal erythrocytes. C) Normal appearance of blood vessels lined with epithelial cells (BV). H&E, Scale bar 25 µm.

The administration of proximol altered spongiotrophoblastic area at some degree in placenta. Common trophoblastic giant cells with degenerative changes were observed. They have many heterogenous cytoplasmic vacuoles with different sizes and are characterized by pyknosis and odd shaped nuclei (Fig. 3A&C).

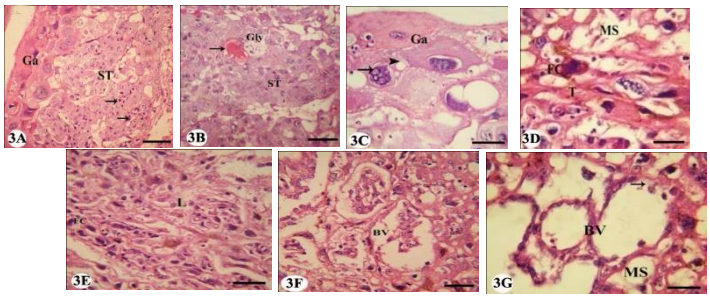

Figure 3: Photomicrographs of a section of placenta of treated pregnant rat. H&E stain. Showing
A) Basal zone; showing increase in number of giant cell (Ga) and Pycnotic cells in spongiotrophoblasts (arrow). ST= spongiotrophoblast. Scale bar:50µm
B) Cystic degeneration of glycogen cells (Gly C) and hemorrhagic area (arrow). Scale bar:25µm
C) Abnormal shape of giant cells like cytoplasmic vacuolation (head arrow) and nuclear vacuolation (arrow). Scale bar: 6.25µm
D) Labyrinth zone; increase in thickness of trophoblastic septa with deposition of fibrin (T) and irregular dilatation of maternal sinusoids (MS). Scale bar: 6.25µm
E) Labyrinth zone (L); showing extensive necrosis. Scale bar:25µm
F&G) Degenerative epithelial cells line the fetal blood vessel in labyrinth zone (BV). Scale bars: 12.5µm &6.25µm respectively

In the basal zone, an increase in spongiotrophoblasts, reduction in clusters of glycogen cells and hyperemia (hemorrhagic cyst) were observed (Fig. 3B). Also, there was an increase in the number of apoptotic cells characterized by pyknosis, karyorrhexis, and phagocytosis.

In the labyrinth area; decreased and irregular vessel formation, an irregular dilatation of maternal sinusoids and increase in the thickness of the trophoblastic trabeculae, were observed (Fig. 3D). Damage in corresponding zone became marked with degeneration and necrosis with accumulation of cell debris was detected. Degenerative epithelial cells line the fetal blood vessel in the labyrinth zone (Fig. E, F&G).

The light microscopic observations revealed that, the placental tissue of the control group showed

positive PAS reaction in the cytoplasm of spongitrophoblast cells of the basal zone, trophoblastic septa and syncytiotrophoblast of the labyrinth zone (Fig. 4A&B). The placental tissue sections of pregnant rats treated with 0.05 mg/Kg of proximol (group B) showed a noticed depletion of the glycogen content in the basal and labyrinth layers (Fig. 5A&B).

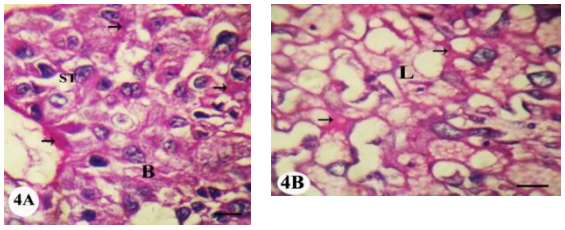

Figure 4: Photomicrographs of a section of placenta of control pregnant rat. Periodic Acid-Schiff's.
A) Showing a positive reaction of PAS in basal zone (arrow), B= basal zone and ST= spongitrophoblast. Scale bar:25µm
B) Showing a positive reaction of PAS in labyrinth zone (L) and (arrow). Scale bar:25µm

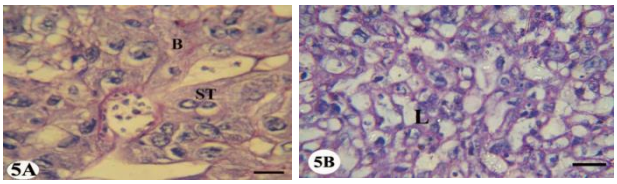

Figure 5: Photomicrographs of a section of placenta of pregnant rat treated with 0.05 mg/Kg of proximol. Periodic Acid-Schiff's.
A) Showing a decrease in glycogen content in basal zone (B), ST= spongiotrophoblast. Scale bar :6.25µm
B) Showing a decrease in glycogen content in labyrinth zone (L). Scale bar 12.5µm

The placental tissue of the control group showed negative reaction of caspase-3 in basal zone, both in giant cells (Fig. 6A) and spongitrophoblast cells (Fig. 6B). It also showed negative reaction in trophoblastic septa and syncytiotrophoblast of the labyrinth zone (Fig. 6C&D). The basal zone of the placenta from animals exposed to 0.05 mg/Kg showed a negative caspase-3 activity in cytoplasm of the gaint cells (Fig. 7A) but there is highly positive caspase-3 in the spongitrophoblast cells (Fig. 7B). The labyrinth zone of the same group showed a highly apoptotic index that scattered in the trophoblastic septa (Fig. C).

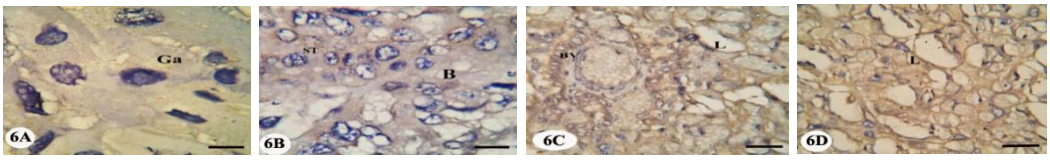

Figure 6: Photomicrographs of a section of placenta of control pregnant rat. Caspase-3 immunostain.
A) Showing negative reaction of caspase-3 of giant cell (Ga). Scale bar:25µm
B) Showing negative reaction of caspase-3 of spongiotrophoblasts (ST) in the basal zone (B). Scale bar:25µm
C&D) Showing negative reaction of caspase-3 of trophoblasts (T) in the labyrinth zone (L). Scale bar: 50µm

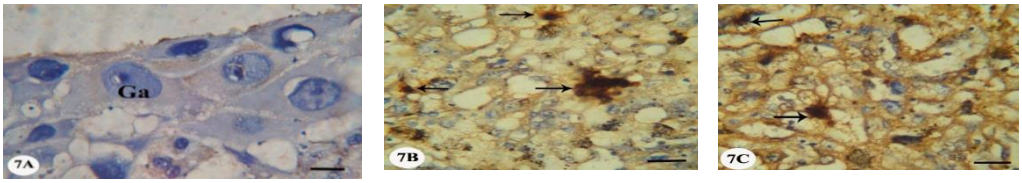

Figure 7: Photomicrographs of a section of placenta of pregnant rat treated with 0.05 mg/Kg of proximol. Caspase-3 immunostain.
A) Showing negative reaction of caspase-3 of giant cell (Ga). Scale bar 6.25µm.
Showing apoptosis (arrow) of spongiotrophoblasts (ST) in the basal zone (B). Scale bar: 50µm
B) Showing apoptosis (arrow) of trophoblasts (T) in the labyrinth zone (L). Scale bar: 50µm

Oxidative stress observations:

CAT serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. Female rats exposed to 0.05 mg/Kg of proximol showed reduction (1.13±0.02 U/g) in the catalase activity when compared with control group (1.67± 0.51 U/g) (Table 2).

Table 2: Showing effect of proximol on placental Catalase and MDA of pregnant rats at 20th day of gestation.

Group	*A*	*B*
Parameter	*(control)*	*(0.05mg/Kg)*
CAT (U/g)	1.67±0.51	*1.13±0.02*
MDA (nmol/g)	114.6±7.4	*302.7±26.2*

Values are expressed as Mean ± SEM. The statistical differences were analyzed by ANOVA followed by independent samples T test. a= $P \leq 0.05$ compared with control. CAT= Catalase, MDA= Malondialdehyde.

The level of MDA was elevated as an indicator of lipid peroxidation. In pregnant rats treated with proximol, significant elevations ($P \leq 0.05$, t= 6.89, df= 2.324) in the levels of MDA were observed (302.7±26.2 nmol/g) when compared to the control group (114.64±7.50 nmol/g).

DISCUSSION

Herbal medicines refer to the use of plants for the promotion of healing and maintenance of health. It is said that the use of herbal medicines originated in Egypt back in 1550 BC. The number of reports of adverse effects of herbal medicines is now increasing due to the awareness among the consumers and clinical practitioners. Thus, the growing use of the traditional herbal drugs around the world requires further scientific investigations about the harmful effect of these herbs. Due to the absence of these knowledge, the present work studied the effects of proximol on the pregnancy.

The placenta is a noticeable organ that allows the exchange of nutrients and metabolic products between the mother and its fetus to ensure convenient fetal growth [19]. The placenta contains highly specialized trophoblast cells that form a barrier between the maternal uterus and the fetus [20]. The growth of this organ is maintained by the maternal blood supply [21], which provides an adequate balance of nutrients, growth factors and hormones [22]. Alterations in this balance may result in several disorders characterized by increased oxidative stress in the placenta, such as preeclampsia, which is believed to result from maternal endothelial dysfunction [23], [24].

The current work showed a reduction in the weight of placenta. A small placenta results in reduced uterine blood flow to the placenta and consequently to the fetuses, which is the major determinant of fetal growth retardation [25].

Phenol is one of the aqueous extract of *Cymbopogon proximus* [3]. Phenols are natural products originated by various plants. They are combustible compounds that are extremely soluble in water, oil and numerous organic solvents. Almost the entire phenols are toxic and some of their compounds are known to be carcinogenic [26], [27]. This compound is sensible hydrophilic and rapidly cross the placenta to transfer into foetus [28]. Also, [29] described opposite effects on progeny development and viability over several generations of rats consuming phenol at concentrations of 7,000 ppm and above in drinking water.

This proves that the phenol can easily cross the placenta and affect the fetuses. Since the phenol is one of the components of proximal, it may be the originator of the placental disorder.

Histopathologically, the labyrinth and basal zones showed an increase in the scattered giant and apoptotic cells, marked decrease in glycogen cell-islands and hypoplasia. In the current study, the treated group showed decrease in amount of carbohydrates in basal and labyrinth layers of the placental tissue as observed by slight reaction for PAS (Periosdic Acid-Schiff's). The decreased carbohydrate contents may be due to disturbance in carbohydrate metabolism or an alteration in some enzymatic activity that led to metabolic degradation and inhibition of the carbohydrate synthesis in the placental tissue.

Apoptosis is a highly regulated and intrinsic cell-suicide program that is important in both physiological and pathological conditions. The central component of apoptosis is a family of proteases called caspases. Cysteine aspartate-specific proteases, known as caspases, are divided into two groups: initiator caspases, such as caspase-8, and -9, whose main function is to activate downstream executioner caspases, such as caspase-3, -6 and -7, which are responsible for degradation of cellular proteins [30]. The present work showed a higher caspase-3 activity in proximol-exposed group than control group.

Reactive oxygen species (ROS) are the by-products of normal cellular oxidative processes, and are generated in the mitochondria and from other sources. ROS are highly reactive and short-lived. They can interact with nearby molecules and thus

inflict serious damage to lipids, proteins and DNA [31]. The present work demonstrates an increase in oxidative stress in placental tissue in response to the herbal drug halfa-bar. These effects can be seen throughout the changes in antioxidant enzyme activity (CAT) and oxidative stress biomarkers (MDA content). This increase in MDA level may be due to the inefficient antioxidant defense system, which is evident by decrease in the activity of catalase. An imbalance between lipid peroxides and the antioxidant system may result in placental dysfunction and subsequent inadequate nutrient transfer to the fetus. The production of lipid peroxides and free radicals by the placenta affects its integrity leading to placental damage as seen in pre-eclampsia [32] or pregnancy complications.

CONCLUSION

Finally, during admistration of poximol (*Cymbopogen proximus*) as herbal drug in pregnancy period, it will pass to embryos through placenta causing several implications to it as shown in our investigation and then embryos. So, poximol should be given with caution.

Financial and conflicts of interest disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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