ADDIS ABABA UNIVERSITY

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EVALUATION OF POSSIBLE TERATOGENIC EFFECTS OF LEAVES OF *MORINGA STENOPETALA* IN RAT EMBRYOS AND FETUSES.

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Evaluation of Possible Teratogenic Effects of Leaves of *Moringa Stenopetala* in Rat Embryos and Fetuses.

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A Thesis Submitted to the Department of Anatomy, School of Medicine, and College of Health Sciences for Partial Fulfillment of the Requirements for Master's Degree in Human Anatomy.

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Declaration

This is to certify that the thesis prepared by Daniel Teshome, titled: Evaluation of possible teratogenic effects of leaves of *Moringa stenopetala* in rat embryos and fetuses at Addis Ababa University on year 2018/2019 and submitted in partial fulfillment of the requirements for the degree of Master of science in Medical Anatomy complies with the regulations of the university and meets the accepted standards with respect to originality and quality. This thesis has not been presented for a degree in any other university, and that all sources of materials used for the thesis have been duly acknowledged.

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LIST OF ABBREVATIONS AND ACRONYMS

AAU:-	Addis Ababa University
ANOVA:-	Analysis of variance
AQ:-	Aqueous
BW:-	Body weight
CHSs:-	College of health sciences
CRL:-	Crown rump length
DPX:-	Dibutyl phythalate in xylene
EPHI :-	Ethiopian public health institute
H&E:-	Haematoxylin and Eosin
M.:-	*Moringa*
OECD:-	Organization of Economic Co-operation and Development
SDM:-	Standard deviation of mean
SNNPR:-	Southern Nation Nationalities and People Region
SPSS:-	Statistical package for social sciences
TMMRD:-	Traditional and Modern Medicine Research Directorate
WHO:-	World health organization

Table of contents

IDENTIFICATION II
AKNOWLEDGMENT IV
LIST OF ABBREVATIONS AND ACRONYMS V
LIST OF TABLES VIII
LIST OF FIGURES VIII
ABSTRACT X
1. INTRODUCTION 1
1.1 Background 1
1.1.1 *Moringa stenopetala* 1
1.2 Statement of the problem 3
1.3 Significance of the study 4
2. LITERATURE REVIEW 5
3. OBJECTIVES 7
3.1 General objectives 7
3.2 Specific objectives 7
4. MATERIALS AND METHODS 8
4.1 Study area 8
4.2 Study design 8
4.3 Study period 8
4.4 Collection of plant 8
4.4.1 Preparation of crude extract 8
4.5 Experimental animals 9
4.5.1 Grouping and dosing of pregnant rats 9
4.6 Day-12 *Moringa stenopetala* leaf extract experiment 10
4.7 Day-20 *Moringa stenopetala* leaf extract experiment 11
4.7.1 External evaluation 12
4.8 Histopathological studies of the placenta 12
4.9 Light microscopy and photomicrography 13
5. DATA PROCESSING AND ANALYSIS 13
6. ETHICAL ISSUES 13
7. OPERATIONAL DEFINITIONS 14

8. RESULTS 16
8.1 Day 12 experiment 16
8.1.1 Pregnancy outcomes 16
8.1.2 Embryonic growth 17
8.1.3 Embryonic development 18
8.1.4 Percentage of retarded development 21
8.2 Day 20 experiment 24
8.2.1 Pregnancy outcomes 24
8.2.2 Fetal growth 25
8.3 Histopathological analysis of placenta 27
9. DISCUSSION 31
10. CONCLUSION 34
11. RECOMMENDATION 35
12. REFERENCES 36
ANNEX 1 41
ANNEX 2 43
ANNEX 3 44

LIST OF TABLES

Table 1: Treatment schedule of day-12 *Moringa* leaf extract experiment..... 11
Table 2: Treatment schedule of day-20 *Moringa* leaf extract experiment..... 12
Table 3: Pregnancy outcome following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment..... 16
Table 4: Embryonic growth following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment..... 18
Table 5: *In vivo* development of rat embryo following treatment with *Moringa* leaf extracts in the day-12 experiment. 20
Table 6: Embryonic circulatory and auditory systems development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment. 22
Table 7: Embryonic nervous system development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment. 22
Table 8: Embryonic craniofacial development after treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment. 23
Table 9: Embryonic musculoskeletal system development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment. 23
Table 10: Daily food intakes and maternal weight gains of animals in day-20 *Moringa* leaf extract experiment..... 24
Table 11: Pregnancy outcomes of day-20 *Moringa* leaf extract experiment. 25
Table 12: Mean fetal growth following treatment with *Moringa* leaf extracts in the day-20 experiment..... 26
Table 13: Percentage of abnormality of vessels within the terminal villi of rat placenta in both control and treatment groups. 28
Table 14: Percentage of decidual hypoplasia and atrophy, and decidual necrosis of rats placenta in both control and treatment groups. 28
Table 15: Percentage of decidual cytolysis, apoptosis, and intervillous space thrombosis of rats placenta in both control and treatment groups. 29

LIST OF FIGURES

Figure 1: *Moringa stenopetala* tree (a), immature pods (b) and leaves (c) grown in Arbaminch area, South Western part of Ethiopia (9). 2

Figure 2**:-** Mean daily food intake in grams of animals from Day 1-5 and Day 6-12 in the day-12 experiment..... 17

Figure 3:- Twelve days old rat embryos inside the yolk sac showing yolk sac circulation..... 19

Figure 4:- Twelve days old rat embryos showing somites, rhombencephalon, mesencephalon and telencephalon. 21

Figure 5:- Twelve days old rat embryos showing heart primordia, forelimb and hindlimb bud, and branchial bars. 21

Figure 6:- Near-term rat fetus. 27

Figure 7: Photomicrograph showing markedly increased sign of revascularization and capillary dilatation (arrows) in M1000mg/kg *Moringa* treated placenta (A) and pair-fed control(B). Stain used H and E, 40X. 29

Figure 8: Photomicrograph showing decidual apoptosis in M1000mg/kg *Moringa* treated placenta (A) and without apoptosis pair-fed control(B). Stain used H and E, 40X..... 30

Figure 9: Photomicrograph showing intervillous thrombosis (arrows) in M1000mg/kg *Moringa* treated placenta (A) and without thrombosis in the pair-fed control placenta (B). Stain used H and E, 40X. 30

Figure 10: Photomicrograph showing a foci of decidual necrosis (arrows) in M1000mg/kg *Moringa* treated placenta (A) and normal decidua in the pair-fed control placenta (B). Stain used H and E, 40.....30

ABSTRACT

Introduction: - *Moringa stenopetala* (Baker f.) is a medicinally important plant, belonging to family *Moringaceae*. This plant has been recently getting great popularity in Ethiopia due to its multiple uses. The leaves of *Moringa stenopetala* are traditionally used for the treatment of various ailments such as malaria, hypertension, and diabetes. The use of *Moringa stenopetala* is known to be beneficial in different systems of the body. However, there have been no studies conducted to investigate its effects on the developing embryo and fetuses.

Objective: - This study was conducted to evaluate the possible teratogenic effects of leaves of *Moringa stenopetala* in wistar rat.

Methods: - The leaves of *moringa stenopetala* were collected in Arbaminch, Southern Ethiopia. The leaves were dried and crushed to powder using mortar and pestle. The dried leaves were extracted by maceration using 80% methanol. The 80% methanolic extract of *Moringa* leaf at doses of 250mg/kg, 500mg/kg and 1000mg/kg were orally administered to pregnant Wistar rats from day 6 through day 12 of gestation. Embryos and fetuses were recovered on gestational day-12 or day-20, respectively, and were quantitatively and qualitatively assessed for developmental anomalies. Histopathological examination was carried out on the placenta from both the treatment and control groups.

Result: -Results of the present study showed that *Moringa* exposure during pregnancy might have teratogenic effects in rat embryos and fetuses at a dose of 1000mg/kg. In the day-20 experiment, significant prenatal growth retardation such as reduced fetal weight and crown-rump length (CRL) were observed in near term fetuses of 1000mg/kg (highest dose) *Moringa* treated animals. This growth retardation was in excess of those in the pair-fed control and *ad libitum* group ($p<0.05$). Fetal weight and CRL in the highest dose treated animals were 2.41±0.108 and 2.81±0.167, respectively. Fetal weight and CRL in the pair-fed control group were 3.11±0.078 and 3.08±0.122, respectively. Growth retardations such as delay in development of otic, optic and olfactory system were also observed in day-12 embryos of 1000mg/kg *Moringa* treated rats. However, in this study, the offspring's of *moringa* treated pregnant rats did not show gross external malformations (neural tube defect missing tail, anencephaly, and polydactyly) at all doses. Sections of the placenta in a high dose treated group whose mother received 1000mg/kg of *Moringa stenopetala* extract showed multiple lesions such as decidual hypoplasia, atrophy and

decidual necrosis in 25% of the placentas. The placenta weight at a high dose treatment and pair-fed control group were 0.459±0.031 and 0.527±0.049, respectively.

Conclusion: -The findings of present study have shown that administration of crude extract of *Moringa stenopetala* at a higher dose was not safe in pregnant Wistar albino rats. Its toxic and teratogenic effects were evidenced by the significant delay in embryonic and fetal development, decrease in maternal weight gain during gestational periods and increase in fetal resorptions and fetal death. Moreover, consumption of *Moringa stenopetala* leaf extract at a high dose had adverse effect on the histology of the placenta as evidenced by intervillous thrombosis, decidual necrosis, and decidual hypoplasia. Therefore, excessive intake of *Moringa stenopetala* leaf may be unsafe.

Keywords: *Moringa stenopetala*, teratogenicity, fetuses, embryo, gestation, Wistar rats.

1. INTRODUCTION

1.1 Background

1.1.1 *Moringa stenopetala*

Moringa stenopetala (Baker f.) is one of the medicinal plants widely used for the treatment of a variety of diseases including hypertension and diabetes mellitus. *M. stenopetala* has belonged to the family *Moringacae,* represented only by a single genus *Moringa.* This genus is represented by 14 species to which *M. stenopetala* belongs (1, 2).

It is often referred to as the African *Moringa* tree because it is native only to Southern Ethiopia and Northern Kenya. However, it grows in many other parts of the tropics, it is not as widely known as its close relative *Moringa oleifera,* but often considered as more desirable than *M. oleifera* (3).

A green, drought-resistant branched *Moringa* tree grows 6 to 10 m tall, thick at base bark with white to pale gray or silvery coloration (4). It grows abundantly in Southwestern Ethiopia at an altitude range of 1000 to 1800m, where the leaves were eaten as a vegetable. It is widely distributed in Konso, Wolayta, Derashe, Gamogofa, Sidama, Bale and Borana areas. It grows well in areas receiving annual rainfall amounts that range from 250 to 1500 mm and between 25 to 35^{o}C, can tolerate up to 48^{o}C in the shade and survive light frost (5, 6).

This species is known by different vernacular names such as “Shiferaw” in Amharic, “Haleko” in Wollaytegna and Gamugna, "Shelchada" in konso and “Cabbage tree” in English (7).

According to Sutherland *et al.,* (1994), the two most common English vernacular names for the tree are ‘drumstick’ (describing the shape of its pods) and ‘horseradish’ (describing the taste of its roots) (8).

M. stenopetala differs from *M. oleifera* because of the leaves are made up of leaflets (3.3-6.5 cm) with a pointed rather than a rounded tip. Its pods are larger than those of *M. oleifera* and twisted when the fruit is fresh. Its seeds are ellipsoidal and not spherical, and cream-colored rather than dark brown (8).

Figure 1: *Moringa stenopetala tree* (a), immature pods (b) and leaves (c) grown in Arbaminch area, South Western part of Ethiopia (9).

The chemical compositions of the leaves of *M. stenopetala* are rutin, 4-(4'-0-Acetyl-L-rhamnosyloxy)-benzylisothiocyanate and 4-(4'-0-Acetyl-L rhamnosyloxy)-benzaldehyde (10) and 0- (rhamnopyranosyloxy) benzyl glucosinolate (11). The leaves of *M. stenopetala* are also contained carbohydrates, crude fibers, vitamins (Vitamin C, ß- carotene) and minerals such as Potassium, Iron, Zinc, Phosphorous and Calcium in significant concentrations (4). Phytochemical screening tests for the hot water infusion and aqueous crude extract of *Moringa stenopetala* leaves revealed that it also contains high (+3) amount of alkaloids, flavonoids, tannins, and saponins (12).

M. stenopetala is a multipurposive plant (4). It is important as human food because the leaves have high nutritional value. It contains high amounts of essential amino acids and vitamins A and C. Gofa, Konso, Burji, and Gamo tribes consume its leaves as a vegetable, especially during the dry season (4, 13).

Usually, people cook the leaves and eat them with their traditional *kurkufa* (cereal preparation from maize and sorghum). The people of Konso and the surrounding communities relay on the plant very much not only for food but also for medicine (7, 14).

Different parts of the plant were studied for nutritional and medicinal values. The leaves are used as vegetable foods (4). The flowers are good nectar sources for honey. The seeds are used in clearing muddy water and the grinded wet or dried root used to treat malaria (7).

It has been reported that *M. stenopetala* has hypotensive (15), antihyperglycemic and hypoglycemic effects (16-19). It has also anti-leishmanial, anti-microbial and anti-fertility

activities (7, 20). Additionally, *M. stenopetala* is important in the treatment of stomach pain and expulsion of retained placenta following birth (oxytocic-like activity) (21).

The beneficial use of *Moringa stenopetala* has been extensively studied and the data have been reported. However; there has not been any study nor data on the teratogenic effects of *Moringa stenopetala* yet. Therefore, the present study is aimed at investigating the possible teratogenic effects of *Moringa stenopetala* in rat embryos and fetuses.

1.2 Statement of the problem

According to the World Health Organization (WHO), more than 4 billion people worldwide (or 80% of the global population) use medicinal plants in primary health care (22). However, although the adverse effects of using medicinal plants are less frequent than those from conventional drugs, some studies have reported that they have adverse effects (23).

Ethiopian plants have shown very effective medicinal value for human and domestic animals (24). This plant-based human and livestock health care persists and remains as the main alternative treatment for different ailments largely due to shortage of pharmaceutical products, prohibitive distance of the health service stations, unaffordable prices by smallholder farmers and pastoralists for conventional drugs, emergence and re-emergence of certain diseases and appearance of drug-resistant microbes (25).

Although the majority of the Ethiopian population (about 80%) relies on traditional remedies as a primary source of health care, toxicity studies of these medicinal plants are limited (26).

M. stenopetala is a medicinally important plant, belonging to family *Moringaceae.* This plant recently has great popularity in Ethiopia due to its multiple uses. However, there is no scientific evidence or research data that shows its effect on the developing embryos and fetuses (27).

Even though there is a general agreement about the diverse biological activity of the different parts of plants belonging to the family *Moringaceae*, there are conflicting reports as to their safety. Taking into consideration the widespread use of *M. stenopetala* as a food and medicinal plant in Ethiopia, and the published evidence of diverse biological activities of extracts from the different parts of a related species, *M. oleifera*, it was felt necessary to investigate the potential teratogenic effects of the extracts on Wistar albino rats. Thus, this study will have the chance to identify the possible teratogenic effects of leaves of *M. stenopetala* in rat embryos and fetuses and its histopathology on the placenta.

1.3 Significance of the study

Studies on the medicinal value of herbal medicines are important to enhance the healthcare situation in Ethiopia where an estimated 80% of people use traditional medicine to meet their primary health care needs. *M. stenopetala* has been used traditionally to treat several human diseases particularly, in some parts of Southern Nation, Nationalities and People Region (SNNPR) of Ethiopia (28). Toxic effects of different substances can result in acute and long-term sequels on maternal health and newborns. Therefore, there is a need to understand the impact of *M. stenopetala* consumption on various body systems including reproductive toxicities. Although there are some studies carried out on medicinal and nutritional values of *M. stenopetala*, the teratogenic effects of the plant have not been studied yet.

The main purpose of this study was to evaluate the possible teratogenic effects of *M. stenopetala* based on different embryonic and fetal developmental parameters. The results of this study will help in the formulation of regulatory legislation regarding the use of this plant. The outcome of the study will serve as a premise for further investigation on this plant and may help to develop intervention strategies to control the use of *M. stenopetala* by pregnant women.

2. LITERATURE REVIEW

No previous studies have been conducted for evaluation of teratogenic disorders associated with administration of *M. stenopetala* extracts. However, several investigations have reported the harmful teratogenic effects of some herbal medicines (29).

A study conducted on plant alkaloids that cause developmental defects through the disruption of Cholinergic Neurotransmission has reported that exposure of a developing embryo or fetus to alkaloids from plants, plant products, or plant extracts has the potential to cause developmental defects in animals (30).

A study done on toxicological evaluations of the crude extracts and fractions of *Moringa stenopetala* leaves in liver and kidney of rats has reported that the percentage of body weight gain was significantly reduced in the highest doses of all extracts (1000 mg/kg/day) compared to normal control in the D15 of the experiment (2).

In a study conducted by chronic administration of butanol fraction of ethanol extract of *M. stenopetala* leaves in alloxan-induced diabetic mice improved the weight gain compared with the diabetic control mice. By the end of the experiment, the BW of the normal control group was significantly increased ($P<0.01$) (17).

In another sub-chronic study, however, it was reported that treatment of rats with aqueous extract of leaves of *Moringa stenopetala* at doses of 500mg/kg and 1500mg/kg increased body weight by 3.6 and 8.2%, respectively (31).

In a sub-acute toxicity study, administration of aqueous crude extracts of *M. oleifera* in day 21 and 28 old rats has resulted in a significant reduction ($p<0.05$) in maternal weight gain of rats administered defatted *Moringa oleifera* extracts at day 21 and 28 as compared to the control group (32). Contrary to this finding, however, other investigators have reported that mice treated with *Moringa stenopetala* at a dose of 900mg/kg body weight (BW) of *M. stenopetala* leaf extract showed a significant increase in BW compared to control group. Although it was not statistically significant, an increase in BW of mice treated at doses of 600 and 750mg/kg BW of *M. stenopetala* leaf extract was also observed (33).

In another study, administration of *Moringa oleifera* extract orally in Charles Foster strain albino rats resulted in an abortifacient activity. In this study, *Moringa oleifera* was administered in aqueous solution at a dose of 175 mg/kg body weight on days 5-10 of gestation. This study reported 100% abortifacient activity of *Moringa oleifera.* The maternal body weight gain was significantly reduced in the treatment group compared to the control group. There was also a high prevalence of fetal resorptions in *Moringa* treated groups (34).

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3. OBJECTIVES

3.1 General objectives

- To evaluate the possible teratogenic effects of leaves of *Moringa stenopetala* in rat embryos and fetuses.

3.2 Specific objectives

- ✓ To evaluate possible teratogenic effects of leaves of *Moringa stenopetala* in Day 12 whole rat embryos.
- ✓ To evaluate possible teratogenic effects of leaves of *Moringa stenopetala* in Day 20 fetuses.
- ✓ To evaluate toxic effects of leaves of *Moringa stenopetala* on the histopathology of the placenta in Day 20 fetuses.

4. MATERIALS AND METHODS

4.1 Study area

The study was conducted at AAU, CHSs, School of Medicine, Department of Anatomy and TMMRD, EPHI.

4.2 Study design

Experimental studies were carried out on 12 days old embryos (Day-12 experiment) and 20 days old fetuses (Day-20 experiment). The first day of confirmed pregnancy was considered as day-0. Animals were treated with a different dose of *M. stenopetala* for a period of 1 week; from day-6 through day-12 of gestation. During the treatment period, the animals were fed on pellets and water. In addition, pregnant animals were treated with *Moringa* leaf extracts. The weight of pregnant animals was recorded either on days 0, 6 and 12 of gestation (for day 12 experiment) or on day 0, 6, 12 and 20 of gestation (for day 20 experiment) (35).

4.3 Study period

The study was conducted from March, 2018 - June, 2019.

4.4 Collection of plant

Fresh *M. stenopetala* leaves were collected based on ethnobotanical description from Southern Ethiopia around Arbaminch, which is about 500km far from Addis Ababa, Ethiopia. The plant material was authenticated by a taxonomist in the EPHI, where a voucher number AL-001 was given and were deposited in the herbarium for future reference (36).

4.4.1 Preparation of crude extract

Fresh *M. stenopetala* leaves were cleaned from any extraneous materials, dried at room temperature, grinded to a powder using mortar and pestle and stored in cool and dry place. Then it was weighed by electronic digital balance and placed in an Erlenmeyer flask, which was wrapped with aluminum foil. The dried leaves were extracted by maceration using 80% methanol and placed in an orbital shaker (DS-500 Orbital Shaker; VWR International, Radnor, PA, USA) at room temperature at 120 rpm for 24 hrs. The methanol extract was filtered using cotton gauze and then with Whatman filter paper No-1. The residue was then re-macerated repeatedly and filtered with Whatman filters paper No-1. The combined filtrates were

concentrated under reduced pressure using Rotavapor (Büchi Rota Vapor R-205, Switzerland) at 40°C. The semi-dried residue was kept on a water bath at 400°C overnight and then with a Lyophilizer (Operan Lyophilizer, Korea) to completely remove the solvent residue. From 960g dry leaf, which was dissolved in 5 liters of 80% methanol in 1 to 5 ratios, 156.14g (16.26%) of crude extract was obtained. Then the dried extract was kept in a tightly sealed container at -20 ºC until use (36).

4.5 Experimental animals

Throughout the course of this investigation, three months old nulliparous female and male Wistar albino rats weighing 200 to 230 were used. The rats used for this study were obtained from the laboratory animal breeding facility of the EPHI. The animals were housed in suspended stainless-steel cages in an environmentally controlled room (22 - 23°C) and relative humidity of 50% ± 10. A cycle of 12hr of light and 12hr of the dark was maintained at all times. During the period of adaptation, all the animals received food and tap water unlimited. The handling of animals and all experimental procedures were carried out according to internationally accepted guidelines (OECD, 2008) (37).

After five days of adaptation period, the animals were mated overnight by placing a male Wistar albino rats into a cage containing two nulliparous female rats. The male rat was introduced into the cage at about 17:00 hours. After an overnight mating, a female rat was inspected for the presence of a copulatory plug the following morning and vaginal smears were taken for microscopic determination of the presence of sperm. The presence of spermatozoa in the vaginal smear was considered as day-0 of gestation (35).

4.5.1 Grouping and dosing of pregnant rats

Pregnant rats were divided randomly into five groups, each comprising 5 animals per group. Group-I was treated with distilled water and served as the pair-fed control group. Group-II, Group-III and Group-IV were treated with 250mg/kg, 500mg/kg and 1000mg/kg of the crude *M. stenopetala* leaf extracts, respectively. Group-V was unrestricted-fed *ad libitum* group. The various doses for the *M. stenopetala* extract were selected based on previous reports of acute toxicity study (2). The *M. stenopetala* extract was weighed and mixed with distilled water and continuously vortexed with a vortex shaker. Final volume was 2ml/100g with the vehicle (distilled water) and oral gavage was used for oral administration (35).

4.6 Day-12 *Moringa stenopetala* leaf extract experiment

This experiment was designed to establish the possible teratogenic effects of *M. stenopetala* in 12 days old whole rat embryos. The experiment was expected to show any growth and developmental anomalies that might not have been apparent in the near-term fetuses due to possible compensatory growth and development. In this experiment, once pregnancy was confirmed, animals were randomly assigned into five groups. Group-I was pair-fed control and served as vehicle (distilled water) treated group. Group-II, Group-III and Group-IV served as experimental groups. Group-V animals served as unrestricted-fed, *ad libitum* group.

Each group was consisted of five pregnant rats. Group-I (Pair-fed control) received distilled water, Group-II (M250) received *moringa* 250mg /kg/day, Group-III (M500) received *moringa* 500mg/kg/day, and Group-IV (M1000) received *moringa* 1000mg/kg/day and Group-V was unrestricted-fed *ad libitum* group. The treatment period was from day 6-12 of gestation. The rationale for administration of the extract from day 6 through day 12 of gestation is because this period represents a period of active embryogenesis and organogenesis (38). Each animal in the respective experimental group (Group II, III and IV) received the control diet of equal amount. The pair-fed control group was taking the same diet in the same amount as the experimental groups and kept in the same environment except the *M. stenopetala* extracts, which were given only for the experimental groups (group-II, III and IV). The daily food intake of each animal was recorded every morning, and animals' weight was recorded on Day 0, 6 and 12 of gestation (35). The grouping and treatment schedule is presented in Table 1.

On day 12 of gestation, at 12:00 hours, the pregnant rats were sacrificed by cervical dislocation and the uterine horns were removed and placed in Hank's balanced salt solution. The uterine horns were then incised along the antimesometrial border to reveal the embryos. With the aid of fine forceps and a dissecting microscope, the membranes surrounding the embryos were removed to reveal the underlying visceral yolk sac. The yolk sac circulation and development were evaluated. The embryos were then explanted and the development of the circulatory, nervous, visual, auditory, olfactory, and skeletal systems, as well as craniofacial development, were assessed quantitatively on the basis of 16 recognizable developmental endpoints (morphological scores), according to the criteria of Brown and Fabro (1981) (39). In addition, the numbers of somites were also counted.

Table 1: Treatment schedule of day-12 *Moringa* leaf extract experiment.

Groups	Number of animals	Treatment
Group I (Pair-fed control)	5	Distilled water + Pair-fed/ restricted-fed
Group II (M250mg/kg)	5	*Moringa* 250mg/kg/day + diet
Group III (M500mg/kg)	5	*Moringa* 500mg/kg/day + diet
Group IV (M1000mg/kg)	5	*Moringa* 1000mg/kg/day + diet
Group V (*Ad libitum*)	5	*Ad libitum* (unrestricted-fed)

4.7 Day-20 *Moringa stenopetala* leaf extract experiment

This experiment was designed to establish the possible teratogenic effects of *M. stenopetala* during gestation in 20 days old rat fetuses. In this experiment, once pregnancy was confirmed, animals were randomly assigned into five groups. Group-I was pair-fed control and served as vehicle (distilled water) treated group. Group-II, Group-III and Group-IV served as experimental groups. Group-V was unrestricted-fed, *ad libitum* group.

Each group was consisting of five pregnant rats. Group-I (Pair-fed control) received distilled water. Group-II (M250) received *moringa* 250mg /kg/day, Group-III (M500) received *moringa* 500mg/kg/day, and Group-IV (M1000) received *moringa* 1000mg/kg/day. Group-V was unrestricted-fed, *ad libitum* group. Each animal in the respective experimental group (Group II, III and IV) received the control diet of equal amount. The pair-fed control group was taking the same diet in the same amount as the experimental groups and kept in the same environment except the *M. stenopetala* extracts, which were given only for the experimental groups (group-II, III and IV). The daily food intake of each animal was recorded every morning, and animals' weight was recorded on Days 0, 6, 12 and 20 of gestation (35).

On a gestational day 20, gravid females were sacrificed by cervical dislocation; the uterine horns were exposed and examined intact. The number of implantation sites was determined by counting the metrial glands, which are yellowish nodules, located along the mesometrial margin of the uterine horns. The metrial nodules, which were not occupied by living or recently dead foetuses, were representing the number of prior resorptions. The numbers of live or dead fetuses were determined by applying gentle pressure on them. The uterine horns were incised along the antimesometrial border to reveal the fetuses, fetal membranes, and the placenta. Fetuses were

then recovered and dissected free of the placenta. The crown-rump length (CRL), litter weight and placental weights were recorded. Following these measurements, the fetuses were fixed in Bouin's solution (aqueous saturated solution of picric acid 75%, formalin 25%, and glacial acetic acid 5%) for gross external examination (35).

Table 2: Treatment schedule of day-20 *Moringa* leaf extract experiment.

Groups	Number of animals	Treatment
G-I	5	Distilled water + Pair-fed/restricted-fed
G-II	5	*Moringa* 250mg/kg/day + diet
G-III	5	*Moringa* 500mg/kg/day + diet
G-IV	5	*Moringa* 1000mg/kg/day + diet
G- V	5	*Ad libitum* (unrestricted-fed)

4.7.1 External evaluation

Fetuses fixed in Bouin's solution were examined head to tail under a dissecting microscope for gross external malformations. The parameters assessed were:

(i) Craniofacial development (exencephaly, anencephaly, microphthalmia, and anophthalmia)

(ii) Development of the limbs (syndactyly, adactyly, polydactyly)

(iii) Vertebral column (neural tube defect, kyphosis, scoliosis)

(iv) Tail development (missing tail); and

(v) External genitalia.

4.8 Histopathological studies of the placenta

Four placentas from each group were randomly selected and put in Bouin's solution. The Bouin's fixed placentas were transferred into 70% ethyl alcohol overnight. Following routine processing for light microscopy, the placenta was blocked in paraffin and cut into sections of 4µm thickness using a Leica rotary microtome (LEICA RM 2125RT, Germany). The sections were mounted on glass slides, stained with hematoxylin and eosin (H&E) and coverslip for microscopic examination. The sections were examined for evidence of structural and vascular alterations using a binocular light microscope (40).

The following structures were examined and used as indices of functional as well as structural changes in the placenta:

a. Basal zone of the placenta
b. Labyrinthine zone
c. Intervillous spaces
d. Giant cells and trophoblasts

4.9 Light microscopy and photomicrography

Stained tissue sections of the placenta were carefully examined by a pathologist under a compound light microscope. Tissue sections from the experimental groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrographs of selected samples of placental sections from both the experimental and control rats were taken under a magnification of x40 objective by using automated built-in digital photo camera (40).

5. DATA PROCESSING AND ANALYSIS

Data was entered using EPI-data Version 3.02 and was exported to SPSS Version 23 for analysis. The data regarding weight gain, pregnancy outcome, fetal growth, and developmental anomalies were analyzed using one-way analysis of variance (ANOVA). The data regarding embryonic development were analyzed by using Chi-Square. Differences at $p<0.05$ were considered as statistically significant.

6. ETHICAL ISSUES

The proposal was submitted to the Research and Ethics Committee of Department of Anatomy School of Medicine, College of Health Sciences, and Addis Ababa University. Following approval by the committee, a letter of cooperation was written to EPHI. Animals used in this study were kept from any unnecessary painful and terrifying situations (OECD, 2008). All animals were given appropriate anesthesia to keep from pathogens, pain and suffering minimal during any surgical operation.

7. OPERATIONAL DEFINITIONS

- ✓ **Reproductive toxicity**: - The occurrence of biologically adverse effects on the reproductive systems of females or males that may result from exposure to environmental agents.
- ✓ **Developmental toxicity**: -The occurrence of adverse effects on the developing organism that may result from exposure prior to conception (either parent), during prenatal development, or postnatally to the time of sexual maturation.
- ✓ **Teratogen: -** is an agent that is able to affect normal development and lead to a congenital anomaly.
- ✓ **Anomaly/malformation: -** A morphologic defect of an organ, part of an organ or larger region of the body that results from an intrinsically abnormal developmental process that occurs during organogenesis.
- ✓ **Implantation (nidation): -**Attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium and its embedding in the endometrium.
- ✓ **Embryo: -** The early or developing stage of any organism, especially the developing product of fertilization of an egg after the long axis appears and until all major structures are present.
- ✓ **Fetus: -** The unborn offspring in the post-embryonic period.
- ✓ **Litter: -** a number of young animals born to an animal at one time.
- ✓ **CRL: -** is the measurement from the vertex of the skull to the midpoint between the apices of the buttocks.
- ✓ **Resorption: -**A conceptus, which has implanted in the uterus, subsequently died and is being, or has been resorbed: Early resorption: evidence of implantation without recognizable embryo/fetus. Late resorption: dead embryo or fetus with external degenerative changes.
- ✓ **Apoptosis: -** is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area that is marked by the fragmentation of nuclear DNA.
- ✓ **Necrosis: -** is a form of cell injury , which results in the premature death of cells in living tissue by autolysis.
- ✓ **Cytolysis: -** is a pathological breakdown of a cell due to the bursting of the cell membrane caused by osmosis.

- ✓ **Angiogenesis:** - is the physiological process through which new blood vessels form from pre-existing vessels, formed in the earlier stage of vasculogenesis. It continues the growth of the vasculature by processes of sprouting and splitting.
- ✓ **Intervillous space:** -is the area between the chorionic plate and the basal plate of the placenta
- ✓ **Intervillous thrombosis** is a discrete focus of coagulated blood in the intervillous space
- ✓ **Trophoblastic giant cell: -** is large, highly polyploid cells that form through the process of endo-reduplication.
- ✓ **Decidua: -** is the modified mucosal lining of the uterus known as the endometrium that forms in preparation for pregnancy
- ✓ **Hypoplasia: -** is underdevelopment or incomplete development of a tissue or organ
- ✓ **Atrophy: -** is the partial or complete wasting away of a part of the body.

8. RESULTS

8.1 Day 12 experiment

8.1.1 Pregnancy outcomes

Treatment of pregnant rats with *moringa* leaf extracts at a dose of 250mg/kg/day, 500mg/kg/day and 1000mg/kg/day from days 6 to 12 of gestation showed that there was a dose dependent decrease in maternal weight gain of dams compared to *ad libitum* and pair-fed control groups. The maternal weight gains at a dose of 1000mg/kg and pair-fed control groups were 4.46±0.61g and 5.93±0.31g, respectively. Body weight was significantly reduced in the *moringa* treated group at the dose of 1000mg/kg compared to all the other groups (Table 3). The number of implantation site did not appear to be different from all the other groups. Regarding fetal resorptions, there was high incidence of fetal resorptions at a dose of 1000mg/kg as compared to all the other groups. Fetal resorption at a dose of 1000mg/kg and *ad libitum* groups were 1.4±0.55 and 0.2±0.45, respectively. Results are summarized in Table 3. Results significantly different ($p < 0.05$) from the *ad libitum* control and pair-fed control groups were identified by ANOVA.

Table 3: Pregnancy outcome following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	Maternal weight gain per dams (g)	Implantation sites per litter	Resorptions per litter
G-I	5.93±0.31	10±1.58	0.4±0.55
G-II	5.51±0.81	9.8±1.3	0.4±0.55
G-III	5.18±1.08	9.8±1.3	0.4±0.55
G-IV	$4.46\pm0.61_a$	8.6±1.14	$1.4\pm0.63_a$
G- V	7.85±1.26	10.2±0.84	0.2±0.45
F – statistics	32.32	1.241	4.071
P – value	<0.001	0.33	0.014

Results are summarized as mean ± SDM

$_a$: significantly different ($p < 0.05$) from *ad libitum*, pair-fed control, M250mg/kg and M500mg/kg groups.

Maternal daily food intake in day 1-5 and day 6-12 in the highest dose treated animals were 15.86g/day and 15.69g/day, respectively. Maternal daily food intake in day 1-5 and day 6-12 in *ad libitum* group were 15.77g/day and 16.01g/day, respectively. Maternal daily food intake was reduced during the treatment period (day 6-12) at the dose of 500mg/kg and 1000mg/kg compared to pair-fed and *ad libitum* control groups (Fig. 2). But, it was not statistically significant.

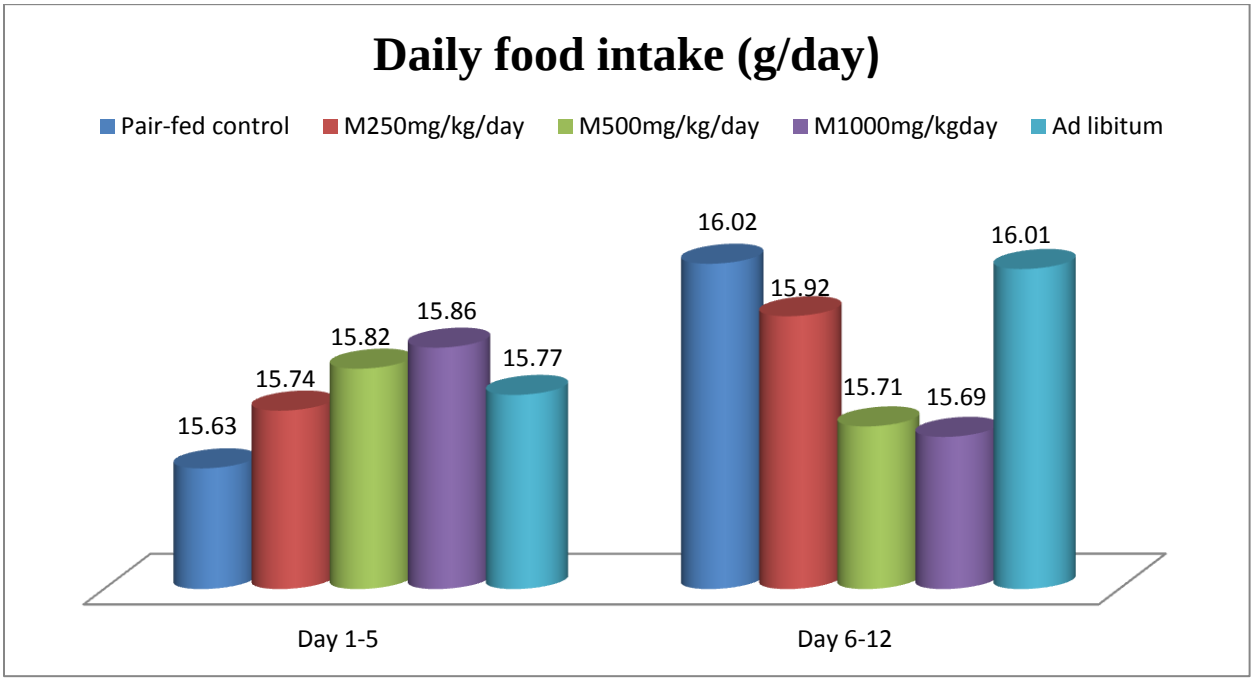

Figure 2**:-** Mean daily food intake in grams of animals from Day 1-5 and Day 6-12 in the day-12 experiment.

8.1.2 Embryonic growth

Embryonic growth indices used were the number of somites and morphological score. The number of somites present was considered as one of the most important criteria for assessing embryonic growth. The number of somites at a dose of 1000mg/kg and *ad libitum* groups were 27.61±1.34 and 30.87±0.93, respectively. Compared to all other groups, the number of somites following treatment with M1000mg/kg *moringa* leaf extract was significantly decreased ($P<0.05$). The morphological score for 1000mg/kg treated group and *ad libitum* groups were 41.74±2.5 and 44.87±2.3, respectively. Embryos of 1000mg/kg *moringa* leaf extract treated group shows a significant reduction in the morphological score compared to all the other groups (Table 4).

Table 4: Embryonic growth following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	Embryonic growth	
	Number of somites	Morphological score
G-I	30.45±1.73	44.64±2.5
G-II	30.73±1.66	44.75±2.4
G-III	30.9±1.43	43.73±2.48
G-IV	$27.61±1.34_a$	$41.74± 2.5_a$
G- V	30.87±0.93	44.87±2.3
F statistic	51.81	11.62
P-value	<0.001	<0.001

Results are summarized as mean ± SDM

$_a$: significantly different ($p < 0.05$) from *ad libitum* control, pair-fed control, M250mg/kg and M500mg/kg groups.

8.1.3 Embryonic development

Developmental status of the primordia of the various systems was assessed according to the morphological scoring system of Brown and Fabro (1981).The results are summarized in table 5.

In rats treated with M1000mg/kg/day *moringa* leaf extracts, the yolk sac of their embryos were not obliterated and vitelline artery and veins were not separated. There was a full yolk sac plexus of vessels (score 3.31±0.47) compared to *ad libitum* group (score 3.54±0.504). The degree of flexion at a dose of 1000mg/kg and *ad libitum* groups were 2.31±0.47 and 2.7±0.465, respectively. An embryo of M1000mg/kg treated group shows a significant decrease in the degree of flexion when compared with pair-fed control, M250mg/kg and *ad libitum* groups.

There were no significant differences in the development of cardiac primordium between the groups (score 3.5). The posterior neuropore was closed in all of the treatment and control groups (score 4). With respect to the closure of anterior neuropore, there were no significant differences between any of the groups (Score 3).

All embryos revealed a completely fused mesencephalon (Score 3). There was no significant difference among the groups. A visible telencephalic evagination was present in all embryos (Score 3).

In *ad libitum* group, there was a well-elevated telencephalic hemisphere (score 4). But, there was no significant difference between all of the groups.

In M500mg/kg/day and M1000mg/kg/day treated groups, there was a significant delay in the development of otic, optic and olfactory systems compared to pair-fed control and *ad libitum* groups. The number of branchial bars in M500mg/kg and M1000mg/kg treated groups were 3.02±0.59 and 2.77±0.43, respectively. The number of branchial bars in pair-fed control and *ad libitum* groups were 3.43±0.542 and 3.46±0.546, respectively. A significant decrease in the number of branchial bars was observed in M500mg/kg and M1000mg/kg treated groups as compared with pair-fed control and *ad libitum* groups. There was also a slight decrease in the number of branchial bars in M250mg/kg treated group but not statistically significant.

The maxillary process was demarcated and visible cleft anterior to the branchial bar I in all of the embryos (Score 1). No significant differences were observed between the groups. In M1000mg/kg/day treated group, there was no sign of mandibular development from the bar I compared to all other groups (Score 0). With respect to forelimb and hind limb development, no significant differences were observed between the groups (Score 2).

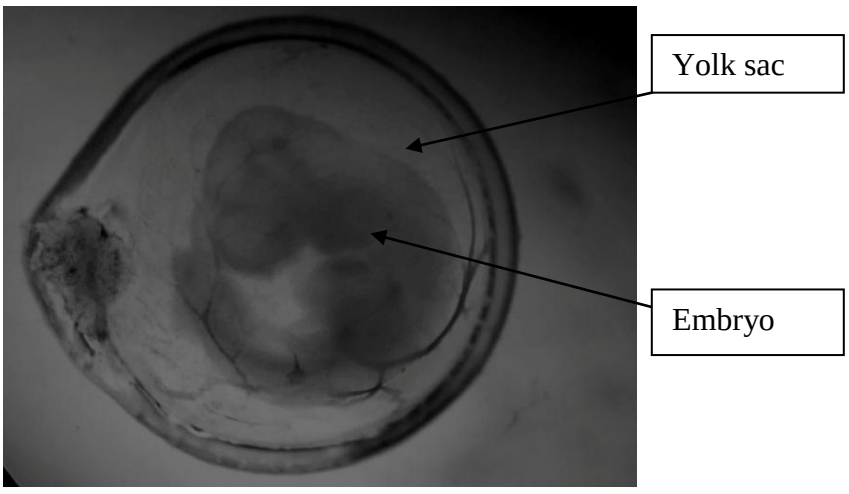

Figure 3:- Twelve days old, rat embryos inside the yolk sac showing yolk sac circulation.

Table 5: *In vivo* development of rat embryo following treatment with *Moringa* leaf extracts in the day-12 experiment.

Morphological end point	G-I	G-II	G-III	G-IV	G-V
Number of fetus/group	47	44	41	39	46
Yolk sac circulation	3.57±0.5	3.59±0.497	3.63±0.488	3.31±0.468$_{a}$	3.54±0.504
Flexion	2.7±0.462	2.68±0.471	2.59±0.499	2.31±0.468$_{a,b,c}$	2.7±0.465
Heart	3.55±0.503	3.55±0.504	3.44±0.502	3.44±0.502	3.57±0.501
Caudal neural tube	4±0.00	4±0.00	4±0.00	4±0.00	4±0.00
Hind brain	3.45±0.503	3.55±0.504	3.37±0.488	3.44±0.50	3.43±0.501
Mid brain	3.49±0.505	3.52±0.505	3.54±0.505	3.33±0.478	3.33±0.478
Fore brain	3.68±0.471	3.66±0.479	3.68±0.471	3.72±0.456	3.74±0.444
Otic system	3.57±0.50	3.49±0.506	3.2±0.553$_{a,b}$	3.15±0.478$_{a,b}$	3.59±0.498
Optic system	2.62±0.491	2.41±0.498	2.07±0.787$_{a,b}$	1.8±0.954$_{a,b}$	2.59±0.498
Olfactory system	0.66±0.479	0.52±0.505	0.41±0.505$_{a,b}$	0.33±0.478$_{a,b}$	0.67±0.474
Branchial bars	3.43±0.542	3.39±0.586	3.02±0.59$_{a,b}$	2.77±0.427$_{a,b}$	3.46±0.546
Maxillary process	1.38±0.491	1.48±0.505	1.41±0.499	1.33±0.478	1.46±0.504
Mandibular process	0.55±0.503	0.58±0.509	0.46±0.505	0.00±0.00$_{a,b,c,d}$	0.54±0.504
Fore limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00
Hind limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00

Statistical differences between the groups were analyzed by Duncan's multiple range tests. Results are expressed as mean ± SDM.

$_{a}$: $p<0.05$, compared to pair-fed control. $_{b:}$ $p<0.05$ compared to the *ad libitum* $_{c:}$ $p<0.05$ compared to M250mg/kg $_{d}$: $p<0.05$ compared to M500mg/kg.

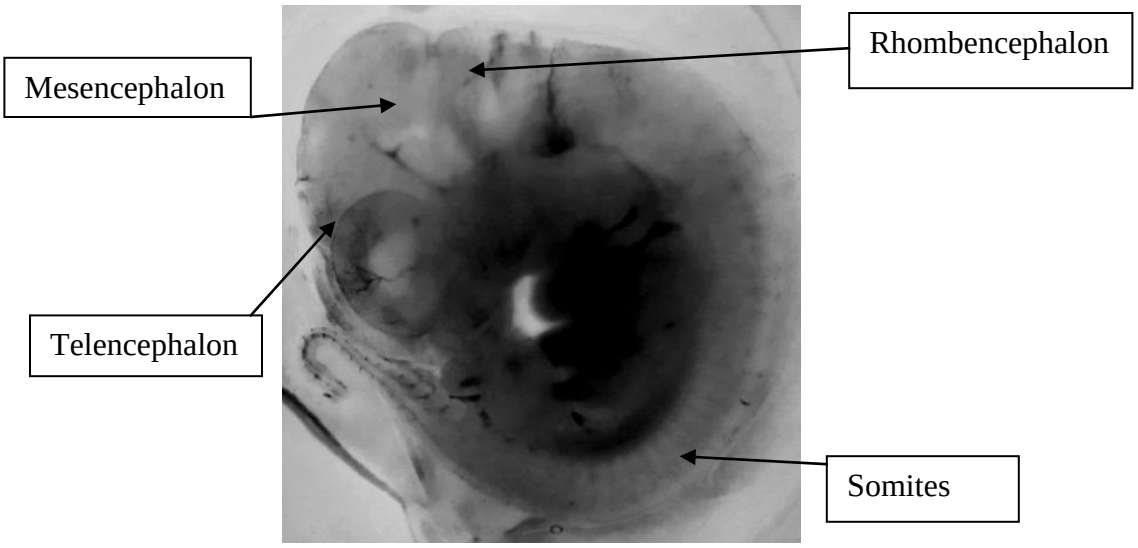

Figure 4:- Twelve days old, rat embryos are showing somites, rhombencephalon, mesencephalon and telencephalon.

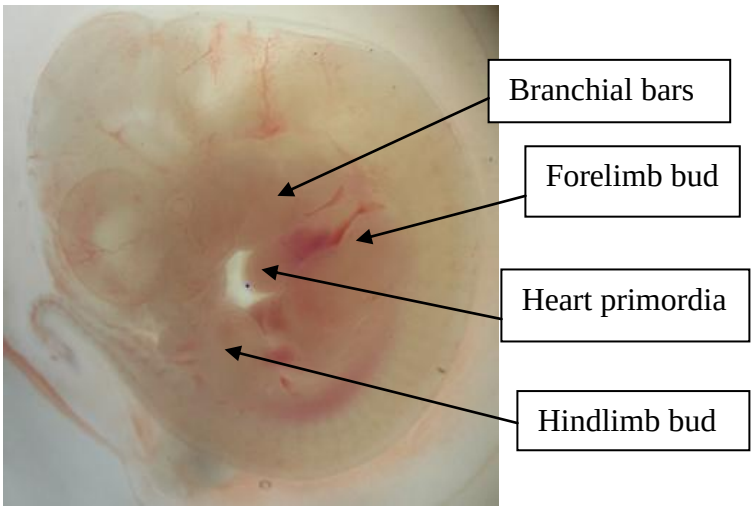

Figure 5:- Twelve days old, rat embryos are showing heart primordia, forelimb and hindlimb bud, and branchial bars.

8.1.4 Percentage of retarded development

The degree of development of yolk sac circulation was retarded by 23.4% in M1000mg/kg treated groups compared to *ad libitum*. The development of the heart was not affected by any of the treatment groups. Otic system development was affected ($p<0.05$) in M500mg/kg and M1000mg/kg treated groups compared to both pair-fed control and *ad libitum* groups.

Table 6: Embryonic circulatory and auditory systems development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	% of retarded development		
	Yolk sac	Heart	Otic system
G-I	3.1	0	2.03
G-II	4.7	0.4	3.6
G-III	9.1	1.2	7.4_a
G-IV	23.4_a	1.1	11.8_a
G- V	0	0	0

Results are summarized as percentages of retarded development.

$_a$: significantly different ($p<0.05$) from all the other groups (Chi-Square).

The forebrain, midbrain, hindbrain and caudal neural tube development were slightly retarded in all treatment groups but not statistically significant (Table 7).

Table 7: Embryonic nervous system development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	% of retarded development			
	Caudal neural tube	Forebrain	Midbrain	Hindbrain
G-I	0	1.8	1.6	0.9
G-II	0	2.6	2.2	1.1
G-III	0	2.1	2.6	2.3
G-IV	0	2.4	2.8	2.1
G- V	0	0	0	0

Results are summarized as percentages of retarded development.

Embryonic craniofacial development and branchial bars ($p < 0.05$) were delayed in both M500 mg/kg and M1000mg/kg treated groups. The development of the forelimb and hind was not affected in any of the treatment groups. The number of somites, the indices of musculoskeletal system development, were significantly affected in M1000mg/kg treated group.

Table 8: Embryonic craniofacial development after treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	% of retarded development			
	Optic system	Olfactory system	Branchial bars	Maxillary process
G-I	0	1.2	0.9	0
G-II	1.1	1.8	3.1	0
G-III	17.67_a	21_a	18.1_a	1.1
G-IV	19.6_a	34.1_a	24.7_a	1.3
G- V	0	0	0	0

Results are summarized as percentages of retarded development.

$_a$: significantly different ($p<0.05$) from all the other groups (Chi-Square).

Table 9: Embryonic musculoskeletal system development following treatment of pregnant rats with *Moringa* leaf extracts in the day-12 experiment.

Groups	Somite number*	% of retarded development	
		Forelimb	Hind limb
G-I	30.45±1.73	0	0
G-II	30.73±1.66	0	0
G-III	30.9±1.43	0	0
G-IV	$27.61±1.34_a$	0	0
G- V	30.87±0.93	0	0

Results are expressed as mean ± SDM (*****) (ANOVA) or as percentages of retarded development

$_a$: Significantly different ($p < 0.05$) from all other the groups (Chi-Square).

8.2 Day 20 experiment

8.2.1 Pregnancy outcomes

Maternal food intake and weight gain

Maternal daily food intake during treatment and post treatment period in the highest dose treated animals were 15.33±0.03g/day and 16.03±0.04g/day, respectively. Maternal daily food intake during treatment and post treatment period in pair-fed control were 15.78±0.32g/day and 16.67±0.35g/day, respectively. During the pre-treatment period (Day 1-5), there were no significant differences in food intake between treatment and control groups. During treatment period (Day 6-12) and post treatment period (Day 13-20), there was dose dependent reduction in daily food intake compared to both pair-fed control and *ad libitum* groups. But, it was not statistically significant.

The maternal weight gains, during the treatment period, in the highest dose treated animals and pair-fed control were 4.41±0.79 and 5.78±0.76, respectively. The maternal weight gain, during the treatment period, in the M1000mg/kg treated group was significantly lower ($p < 0.05$) compared to both pair-fed control and *ad libitum* groups. Significant reductions in maternal weight gains were also seen in M500mg/kg and M1000mg/kg treated groups during day 6-20 compared to both pair-fed control and *ad libitum* groups.

Table 10: Daily food intakes and maternal weight gains of animals in day-20 *Moringa* leaf extract experiment.

Groups	Daily food intake (g/day)			Maternal weight gain (g/day)	
	Day 1-5	Day 6-12	Day 13-20	Day 6-12	Day 6-20
G-I	15.19±0.17	15.78±0.32	16.67±0.35	5.78±0.76	15.87±0.81
G-II	15.18±0.18	15.46±0.12	16.22±0.21	6.47±0.67	16.06±0.76
G-III	15.17±0.15	15.34±0.15	16.13±0.08	5.83±0.83	14.15±0.56
G-IV	15.37±0.07	15.33±0.03	16.03±0.04	4.41±0.79$_a$	10.73±0.57$_a$
G- V	15.31±0.08	15.79±0.15	16.75±0.42	6.89±0.67	18.48±0.85
F-statistic	2.16	8.44	78.72	12.26	84.98
P-value	0.111	0.072	0.061	<0.001	<0.001

Results are expressed as mean ± SDM.

$_a$: Results significantly different ($p < 0.05$) from both *ad libitum and* pair fed-control group (ANOVA).

The numbers of fetuses in M250mg/kg, M500mg/kg and M1000mg/kg moringa treated groups were 43, 43 and 41, respectively. There was a dose-dependent reduction in the number of fetuses and implantation sites among the groups but not statistically significant. With respect to fetal resorptions, there was a high incidence of fetal resorptions in M1000mg/kg treated groups compared to all the other groups. Fetal resorption at a dose of 1000mg/kg and *ad libitum* groups were 1.6±0.55 and 0.2±0.45, respectively. The number of live fetuses in 1000mg/kg *moringa* treated group and *ad libitum* groups were 7.6±0.89 and 9y±0.71, respectively. It was significantly decreased in M1000mg/kg treated groups compared to both pair-fed control and *ad libitum* groups. Compared to all other groups, there was also a high incidence of fetal death in this group (M1000mg/kg). But, the relationship was not statistically significant at the level of 0.05 (ANOVA).

Table 11: Pregnancy outcomes of day-20 *Moringa* leaf extract experiment.

Groups	No. of fetuses	Implantation sites	Number of resorptions/litter	Number of live fetus/dam	Number of dead fetus/dam
G-I	49	9.2±0.84	0.42±0.52	9.1±0.89	0.4±0.55
G-II	43	8.6±0.89	0.45±0.51	8.4±0.55	0.33±0.45
G-III	43	8.8±0.84	0.44±0.51	8.4±0.55	0.4±0.55
G-IV	41	8±1.22	$1.6\pm0.55_{a}$	$7.6\pm0.89_{b}$	0.7±0.447
G- V	48	9.4±1.14	0.42±0.52	9±0.71	0.4±0.55
F statistic	0.841	2.46	4.8	4.333	0.923
P value	0.501	0.079	0.007	0.011	0.47

Results are summarized as mean ± SDM

$_{a}$: Significantly different ($p < 0.05$) from *ad libitum*, pair-fed control groups, M250mg/kg, M500mg/kg and M1000mg/kg groups (ANOVA).

$_{b}$: significantly different($p<0.05$) from *ad libitum* and pair-fed control groups.

8.2.2 Fetal growth

The litter weight of M1000mg/kg treated group and *ad libitum* group were 2.41±0.108 and 3.08±0.093, respectively. The growth of fetus at term was significantly ($p<0.05$) affected at the highest doses of treatment groups compared to the pair-fed control and the unrestricted *ad*

libitum group. The CRL of M1000mg/kg treated group and *ad libitum* group were 2.81±0.167 and 3.08±0.104, respectively. It was significantly lower in M500mg/kg and M1000mg/kg treated groups compared to the pair-fed control and the unrestricted *ad libitum* group. The placental weight of M1000mg/kg treated group and pair-fed control group were 0.459±0.031 and 0.527±0.049, respectively. It was also significantly decreased in M1000mg/kg treated group compared to all the other groups.

Table 12: Mean fetal growth following treatment with *Moringa* leaf extracts in the day-20 experiment.

Groups	Fetal growth		
	Litter weight/fetus(g)	CRL/fetus (cm)	Placental weight/fetus (g)
G-I	3.11±0.078	3.08±0.122	0.527±0.049
G-II	3.04±0.093	3.03±0.141	0.529±0.042
G-III	$2.95±0.117_a$	$2.98±0.125_a$	0.528±0.038
G-IV	$2.41±0.108_a$	$2.81±0.167_a$	$0.459±0.031_b$
G- V	3.08±0.093	3.08±0.104	0.549±0.041
F statistic	367.4	30.9	47.1
P value	<0.001	<0.001	<0.001

Results are summarized as mean ± SDM.

$_a$: Significantly different ($p<0.05$) from *ad libitum* and pair-fed control groups. $_b$: Significantly different ($p<0.05$) from *ad libitum,* pair-fed control groups, M250mg/kg and M500mg/kg groups (ANOVA).

Gross external developmental anomalies were not seen on craniofacial development, development of the limbs, vertebral column; like neural tube defect, tail development (missing tail) and external genitalia.

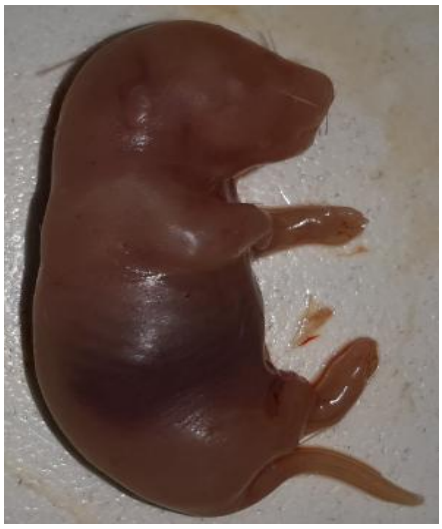

Figure 6:- Near-term rat fetus.

8.3 Histopathological analysis of placenta

Gross anomaly was not observed during the external examination of a placenta in both treatment and control groups. But, there was a significant reduction in placental weight of pregnant rats that received a high dose (M1000mg/kg) of *moringa* leaf extract as compared to all the other groups.

From the histological observation of sections of placenta after staining with Haematoxylin and Eosin, different zones of the placenta were observed. It shows that there was some structural change in decidua basalis, trophoblastic zone and labyrinthine zones of the placenta.

Based on the table shown below, 25 % of terminal villi vessels were dilated in both M500mg/kg and M1000mg/kg *moringa* treated groups, whereas in other groups there was no terminal villi dilatation in the trophoblastic zone of a placenta. In M500mg/kg and M1000mg/kg *moringa* treated groups, 50% of the placenta had an increase in a number of terminal villi vessels, but in the rest of the group's vascular proliferation accounts for 25%.

Table 13: Percentage of abnormality of vessels within the terminal villi of rat placenta in both control and treatment groups.

Groups	% of Abnormality of vessels within terminal villi			
	Capillary dilatation		Terminal villi proliferation	
	Present	Absent	present	Absent
G-I	0	100	25	75
G-II	0	100	25	75
G-III	25	75	50	50
G-IV	25	75	50	50
G- V	0	100	25	75

Sections of the placenta in high dose group whose mothers received 1000mg/kg of *Moringa stenopetala* extract showed multiple lesions that include decidual hypoplasia and atrophy, cytolysis, apoptosis and decidual necrosis in 25% of the placentas. Of M1000mg/kg *moringa* treated placenta, 25% had intervillous thrombosis, but it was not observed in the rest of *moringa* treated and control groups. There were no noticeable changes in the glycogen cells, spongiotrophoblast and trophoblastic giant cell in all of the treatment and control groups.

Table 14: Percentage of decidual hypoplasia and atrophy, and decidual necrosis of rats placenta in both control and treatment groups.

Groups	% of decidual hypoplasia and atrophy		% of decidual necrosis	
	Present	Absent	Present	Absent
G-I	0	100	0	100
G-II	0	100	0	100
G-III	0	100	0	100
G-IV	25	75	25	75
G- V	0	100	0	100

Table 15: Percentage of decidual cytolysis, apoptosis, and intervillous space thrombosis of rats placenta in both control and treatment groups.

Groups	% of decidual cytolysis		% of decidual apoptosis		% of Intervillous Space thrombosis	
	Present	Absent	Present	Absent	Present	Absent
G-I	0	100	0	100	0	100
G-II	0	100	0	100	0	100
G-III	25	75	0	100	0	100
G-IV	25	75	25	75	25	75
G- V	0	100	0	100	0	100

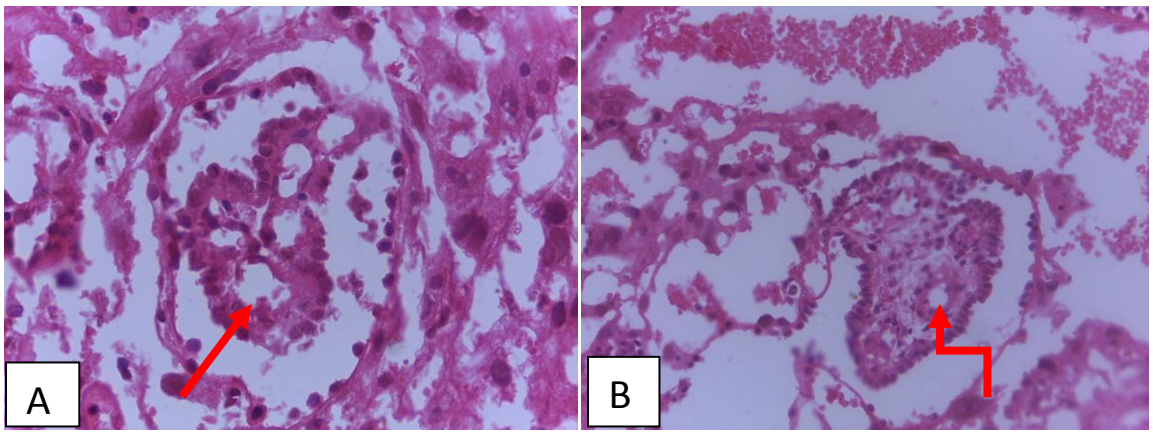

Figure 7: Photomicrograph showing markedly increased sign of revascularization and capillary dilatation (arrows) in M1000mg/kg *Moringa* treated placenta (A) and pair-fed control(B). Stain used H and E, 40X.

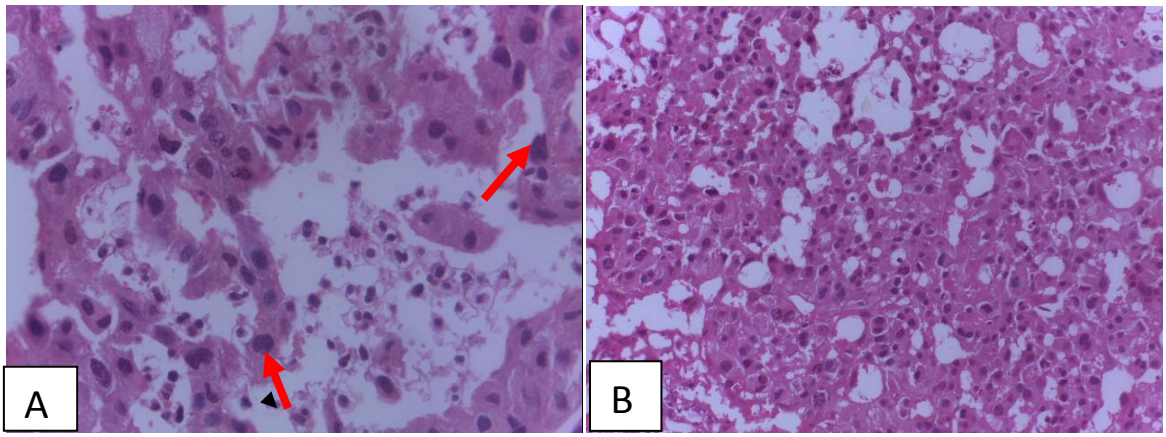

Figure 8: Photomicrograph showing decidual apoptosis in M1000mg/kg *Moringa* treated placenta (A) and normal decidua in pair-fed control(B). Stain used H and E, 40X

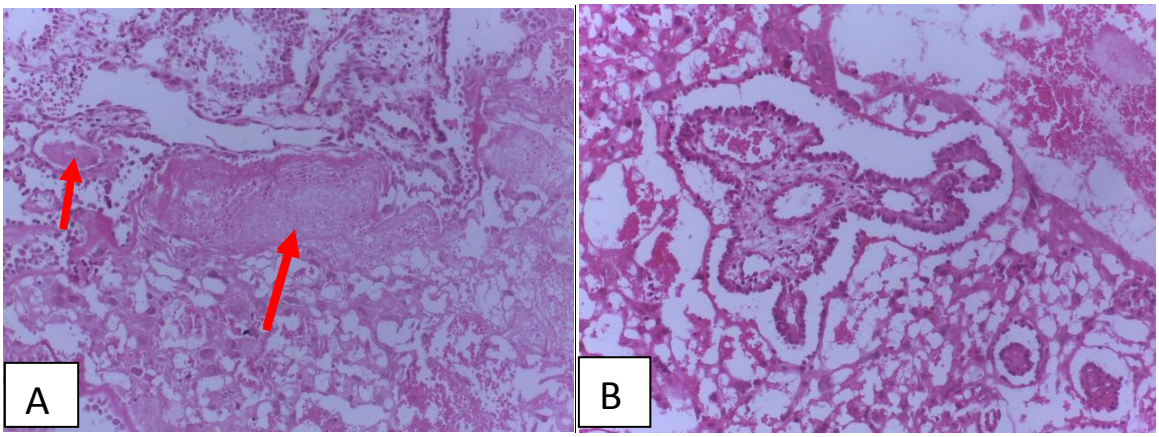

Figure 9: Photomicrograph showing intervillous thrombosis (arrows) in M1000mg/kg *Moringa* treated placenta (A) and without thrombosis in the pair-fed control placenta (B). Stain used H and E, 40X.

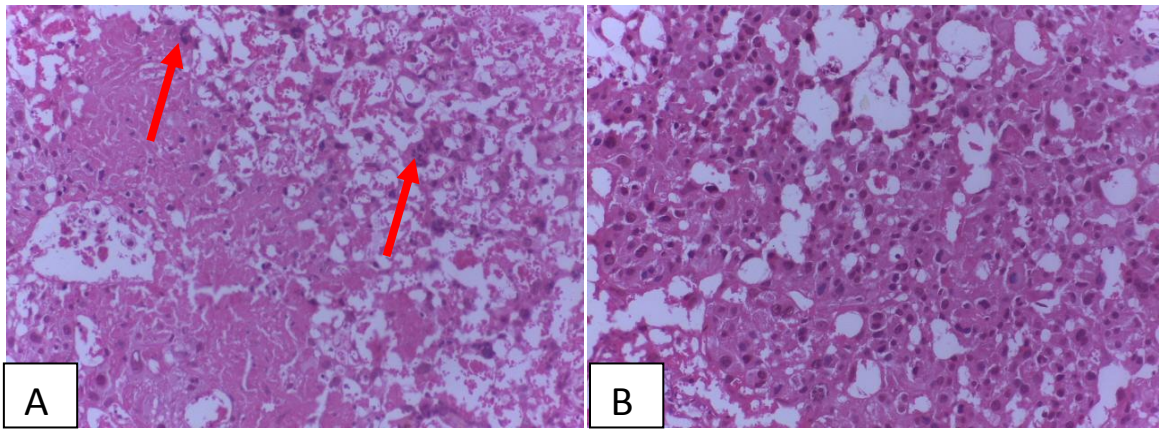

Figure 10: Photomicrograph showing a foci of decidual necrosis (arrows) in M1000mg/kg *Moringa* treated placenta (A) and normal decidua in the pair-fed control placenta (B). Stain used H and E, 40X.

9. DISCUSSION

The utilization of herbal medicines and phytonutrients/nutraceuticals continues to rise as a result of increased acceptance and public interest in both developed and developing nations (41). Herbs and natural plant products are particularly gaining popularity for the management of cardiovascular diseases and associated disorders (42). The soaring interest in traditional medicine is attributed to the failure of modern medicine to alleviate many chronic illnesses.

M. stenopetala, a plant highly venerated for its nutritional values and medicinal properties, it is used in the management of hypertensive and kidney-related disorders (43). However, it is still unknown what effect *Moringa* may have on the developing embryo and fetus.

Administration of *Moringa stenopetala* at the highest dose (1000 mg/kg/day) showed a significant reduction in maternal weight gain compared to both pair-fed control and *ad libitum* groups. This finding is in line with the reports of previous studies conducted on toxicological evaluations of the crude extracts and fractions of *M. stenopetala* leaves. This study also revealed a significant reduction in maternal weight gain at the same doses of *Moringa stenopetala* (1000mg/kg were used) (2). Our study finding is also in agreement with another investigation on sub-acute toxicity of crude extracts of *M. oleifera* in rats (32).

However, our findings were contrary to reports of a study conducted on the chronic effects of *moringa stenopetala* on blood parameters and histopathology of liver and kidney in mice (33). This discrepancy may be due to variation in animal model and duration of administration of the extract.

In the present study, the number of implantation site did not appear to be different from all the other groups. But, the incidence of fetal resorption was higher in M1000mg/kg treatment group compared to *ad libitum* group. This finding was in agreement with the data reported by a study conducted on the abortifacient activity of *Moringa oleifera* in rats (34). This effect may be explained by the fact that the active compounds of the plants are the same in both species.

Embryonic growth was assessed by counting number of somites and morphological scores. In the current study, there was a significant reduction in number of somites and morphological scores at the highest dose (1000mg/kg/day) compared to all the other groups.

Embryonic developments were also assessed according to the morphological scoring system of Brown and Fabro (1981) to examine the expected development of different systems at the end of organogenesis (39). The results showed that a statistically significant delay in the development of the embryo was observed at the doses of 1000mg/kg/day *moringa* leaf compared to both pair-fed control and *ad libitum* groups. Delayed development of yolk sac, otic system, optic system, and olfactory system were also observed at this dose (1000mg/kg).

Regarding the craniofacial development, there was a significant reduction in the degree of flexion, reduction in number of branchial bars, and delay in development of a mandibular process in M1000mg/kg/day treated group compared to both pair-fed control and *ad libitum* groups. This may be due to the presence of alkaloids in *moringa* leaf extracts which may result in developmental delay (30).

But, the development of primordia of heart, forelimb, hind limb and development of the central nervous system including caudal neural tube, forebrain, midbrain, and hindbrain were not different among various groups.

Prenatal growth retardation such as reduced litter weight and crown-rump length were observed in near term fetuses of high dose (1000mg/kg/day) of *moringa* leaf extract treated animals compared to those in the pair-fed and *ad libitum* control groups. In addition, there were also statistically significant increment in frequencies of fetal resorption per litter and number of dead fetuses. The placental weight was also significantly lower in M1000mg/kg/day treated groups compared to all the other groups.

The present study has also revealed a dose-dependent decrease in number of fetuses per dam but this was not statistically significant. However, developmental anomalies and gross external organ malformations were not observed in all fetuses of animals treated with *moringa* at all doses.

The placenta is a temporary structure unique to pregnancy functions to sustain and protect the fetus until birth (44). It obtains its metabolic, immunological requirements (45) and secretory functions to support fetal development. The placenta is attached to the uterus, and the fetus is connected to the placenta via the umbilical cord (46).

Findings from our study showed that administration of high dose(1000mg/kg) of *Moringa stenopetala* leaf extract to Wistar albino rats produce some structural changes in the placental histology.

The terminal blood vessels were dilated in 25% of M1000mg/kg *moringa* treated placenta. There was also an increase in number of terminal villi in 50% of the placenta of M1000mg/kg *moringa* treated group. Intervillous thrombosis, decidual necrosis, apoptosis, cytolysis, and decidual hypoplasia and atrophy were also seen in 25% of placental tissue administered with a high dose of 1000mg/kg of *Moringa stenopetala* leaf extract. This may be due to the presence of flavonoids which is one of the chemicals found in *moringa stenopetala* is also known for its anti-cancer properties to be responsible for the apoptosis of the placenta (47).

Although the mechanism (s) of *M. stenopetala* toxic effects on the placentas is not clear, the leaf of this plant contains different active components including alkaloids, saponins, tannins, and flavonoids that could be responsible for these changes.

It has been suggested that trophoblastic giant cells participate in a number of processes essential to a successful pregnancy including blastocyst implantation, remodeling of the maternal decidua and secretion of hormones that regulate the development of both the fetal and maternal of the placenta.

It is also known that trophoblasts in the fetal part of the placenta are a common toxicological target tissue for some drugs and chemicals because they have high proliferative activity and constitute a major structural component of the fetal part of the placenta (47). However, in the current study, there were no noticeable changes in the glycogen cells, spongiotrophoblast and trophoblastic giant cell in all of the treatment and control groups.

10. CONCLUSION

In conclusion, results of the present study have shown that administration of crude extract of *Moringa stenopetala* at a higher dose was not safe in pregnant Wistar albino rats. Its toxic and teratogenic effects were evidenced by the significant delay in embryonic and fetal development, decrease in maternal weight gain during gestational periods and increase in fetal resorptions and fetal death. Moreover, consumption of *Moringa stenopetala* leaf extract at a high dose had adverse effect on the histology of the placenta as evidenced by intervillous thrombosis, decidual necrosis, and decidual hypoplasia. Therefore, excessive intake of *Moringa stenopetala* leaf may be unsafe.

11. RECOMMENDATION

❖ Based on this study, the following are recommended:

- ✓ Pregnant women may have to take precautionary measures in consuming large amount of leaves of *Moringa stenopetala*.
- ✓ Higher doses of *Moringa* should be investigated in order to determine any effects on other organs such as the brain.
- ✓ Further studies are recommended to investigate the effects of *Moringa stenopetala* during early periods of gestation.
- ✓ Treatment of test animals should also extended to the full gestational period.
- ✓ Extended first-generation and second-generation reproductive toxicity should be conducted.
- ✓ Further investigation should also be carried out using advanced technologies to isolate and identify the active ingredient present in the leaves of *M. stenopetala* and to study the mechanism of action of *Moringa* during pregnancy.
- ✓ Experiments should also be conducted with non-rodent species.
- ✓ Serum level determination of *Moringa stenopetala* extract is recommended.
- ✓ Conducting further study by increasing the number of animals per group is recommended.

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ANNEX 1

MORPHOLOGICAL SCORING SYSTEM FOR RAT EMBRYOS

Morphological end point	0	1	2	3	4	5	Scores
Yolk sac circulation system	not visible or scattered island	corona of blood islands with/without anastomosis	vitelline vessel with few yolk sac vessels	full yolk sac plexus of vessels	yolk sac obliterated vitelline artery and vein well separated		
Flexion	ventrally convex	Turning	dorsally convex	dorsally convex with spiral torsion			
Heart	endocardial rudiment not visible or visible but not beating	beating "s "shaper cardiac tube	convoluted cardiac tube	bulbus cordis,atrium commune or ventriculurcommunes	dividing atrium communes		
Caudal neural tube	Neural plate or fold	closing but unfused neural fold/groove	neural fold closed at level of somite4/5	posterior neuropore formed but open	posterior neuropore closed		
Hind brain	Neural plate	rhombomere A and B	anterior neuropore formed but open	anterior neuropore closed rhomboncephalon formed	pronounced pontine flexurewith transparent roof of 4th ventricle		
Midbrain	Neural plate	mesencephalic brain folds	closing of mesencephalic folds	completely fused mesencephalon	visible division b/n mesencephalon and diencephalon		
Forebrain	Neural plate	prosencephalic brain folds	completely fused prosencephalon	visible telencephalic evagination	well elevated telencephalic hemisphere		
Otic system	no sign of otic dev't	flattened otic primordium	otic pit	Otocyst	otocyst with dorsa recess	otocyst with endolymphatic duct	

Optic system	no sign of optic dev't	sulcus opticus	elongated optic primordium	primary optic vesicle with open optic stalk	indented lens plate	lens pocket or vesicle	
Olfactory system	No sign of olfactorydev't	olfactory plate	olfactory plate with rim	distinct olfactory ridge	lateral nasal process and medial rim		
Branchial bars	none visible	I visible	I and II visible	I, IIand II visible	II overgrowing and obscure III		
Maxillary process	No sign of maxillarydev't	Maxillary process demarcated. Visible cleft anterior to bar I	Maxillary process fused with nasal process				
Mandibular process	No sign of mandibular dev't from bar I	First branchial bar fused and forming mandibular process					
Fore limb	No sign of fore limb dev't	Distinct evagination of wolfian crest at the level of somite 9-13	Forelimb bud	Paddle shaped for limb bud	Distinct apical ridge on forelimb		
Hind limb	No sign of hind limb dev't	Distinct evagination of Wolfian crest at level 01 somite's 26-30	Hindlimb bud	Paddle shaped for hind bud			
Somite's	0-6	7-13	14-20	22-27	28-34	35-41	

ANNEX 2

CHECKLIST RELATED TO PLACENTAL HISTOPATHOLOGY

Slide code	**A**		**B**	**C**	**D**	**E**	**F**	**G**	**H**
	Abnormalities of blood vessels within the villi		Apoptosis	cytolysis	Intervillous thrombosis	Decidual hypoplasia & atrophy	necrosis	Giant cell abnormality	Remark
	Lumen dilation **(Y/N)**	Terminal villi proliferation **(Y/N)**	**Y/N**	**Y/N**	**(Y/N)**	**(Y/N)**	**Y/N**	**Y/N**	
1									
2									
3									
4									
5									
6									
. . . .									

ANNEX 3

I. METHOD OF TISSUE PROCESSING

1. **Fixation:-** The tissue was preserved/fixed in 10% formalin solution
2. **Dehydration:** - The preserved tissue was washed in running tap water for 4-6 minutes. Then, they passed through upgraded alcohol as follows:- 70% alcohol – 1hour, 85 % alcohol – 1hour, 96% alcohol – 1hour, Absolute alcohol I– 1hour, Absolute alcohol II – 1hour.
3. **Clearing: –**Clearing of tissue was done in xylene, -1hour in xylene -I, then after in xylene II for 1 hour.
4. **Infiltration:** -Tissue was infiltrated with paraffin wax I, for 1and 1/2 hrs, paraffin wax II for 2 and 1/2 hrs and paraffin wax III for overnight.
5. **Embedding: -** The cleared tissue was put in molten wax (melting point 56-58degree Celsius) for 12 hours in cryostat. The paraffin blocks of tissue were made with the help of embedding cassettes.
6. **Sectioning: –** The serial paraffin sections of 5-micron thickness was cut by rotator microtome and floated in water bath having temperature 45-50 degree Celsius. The section was made spread on the slide smeared with adhesive solution (mixture of equal amount of glycerol and egg albumin). The slide was dried on hot plate having temperature of 50 ºC.
7. **Deparaffinization of sections: –** The slide was put in xylene II, changes each for 5-10 min in order to remove the extracellular and intracellular wax.
8. **Rehydration: -** The slide was put in descending grades of alcohol i.e. absolute, 90 %, 70 % and 50% alcohol for 2 min for each. The slide was then washed in running tap water for 2 minute and then taken for routine H & E staining.

II. METHOD OF STAINING

a. Stained with Hematoxylin for 10 minutes.

b. Washed in running tap water until section become blue.

c. Stained in 1% eosin for 7- 10 min.

d. Washed in running tap water (5 minute)

e. Dehydrated through 70 % and 95% Alcohol 3 minutes for each, then absolute alcohol I and Absolute alcohol II for 1 and 1/2 hour for each.

f. Cleaned by - Xylene I and Xylene II,5 minutes for each.

g. Mounted – By DPX