Effect of *Macrotyloma uniflorum* (Horse gram) on early and mid-pregnancy of rats

Ruwini Wijenayake, Gayani Galhena and WD Ratnasooriya

Abstract

*Macrotyloma uniflorum* (Horse gram) is a popular pulse in Asia. This study has investigated the effect of aqueous seed extract of horse gram on early and mid-pregnancy using female Wister rats. The extract or distilled water was orally administered during day 1-7 or day 8-14 of pregnancy as daily single doses. Oral administration of the extract (6.17 g/kg) in early pregnancy has significantly increased the pre-implantation loss compared to the controls (28.96 ± 2.26% vs 45.44 ± 5.42%; *P* < 0.05) with a decrease in implantation index (71.04 ± 2.26% vs to 54.56 ± 5.42%). The administering of the extract in mid pregnancy significantly reduced the weight, tail length, and craniosacral length of litter. This shows that consumption of horse gram in early pregnancy can interfere with successful implantation of embryo, while in mid pregnancy, it induces retardation of pup development.

Keywords: *Macrotyloma uniflorum*, pregnancy, implantation, growth retardation

1. Introduction

*Macrotyloma uniflorum* (Horse gram) is a popular pulse, belonging to Family Fabaceae. It is well-known as a medicinal plant in indigenous medicine. Different parts of the plant such as leaves, seeds, and flowers are used in treating various heart conditions, asthma, bronchitis, urinary discharges, kidney stones and diabetics [1, 2]. Since *M. uniflorum* is considered as a nutrient food which gives energy, pregnant mothers tend to consume *M. uniflorum* considering benefits to both her and baby.

However, the aqueous seed extract (ASE) of *M. uniflorum* is claimed to be used in folk medicine to eliminate unwanted pregnancies at their early stages. If such abortive effect exists, it might cause damages to fetus despite the nutritional value. Nevertheless, this abortive effect has not been scientifically investigated so far and hence the present study aims to investigate the effect of aqueous seed extract (ASE) of *M. uniflorum* on pregnancy using a rat model.

2. Methods

Ethical clearance for the study was obtained from the Ethical Review Committee of the Institute of Biology, Sri Lanka (ERC IOBSL145 03 16). ASE was prepared using standard decoction preparation method (60 g of seeds was boiled in 1920 mL of water until a final 240 mL volume of extract was obtained). Human equivalent dose (HED) was converted in to Animal Equivalent Dose (AED) using a standard equation [3].

Wistar rats (*Rattus norvegicus*) were acclimatized to the novel environment one week before the experimentation. Pregnant Wister rats of five to six months old were used for the experiment. Weight of each rat was recorded using an electronic animal balance (MP 600, Chyo, Japan). Rats were observed daily for any possible vaginal bleeding signs starting from 1st day of pregnancy until the laparotomies were conducted.

The effect of ASE on early early and mid pregnancies

Two separate experiments were conducted to test the effect of ASE on early and mid pregnancies. To test the effect on early pregnancy, fourteen pregnant female Wister rats were randomly assign to two groups as control and treatment (*n* _control_ =6, *n* _treatment_ =8). Treatment group was administered with 1 mL of 6.17 g/kg (HED) of extract while control group was administered with 1 mL of distilled water for 1-7 days of the pregnancy at a specific time of each day (1000-1100h). On the 10th day of pregnancy, two groups were laparotomized under di-ethyl ether using aseptic conditions.

The effect on mid pregnancy was tested in two randomly selected groups of female rats (*n* =6). They were orally administered either with distilled water or ASE (6.17 g/kg) from 8th day to 14th day of pregnancy at a specific time of each day 1000-1100h. On the 15th day of pregnancy, the two groups were laparotomized under di-ethyl ether using aseptic conditions.
Laparotomy was conducted according to the standard guidelines for animal surgery. During the laparotomy, the uterus was examined in situ for the total number of uterine implants, the number of viable implants, the number of dead implants and the diameter of the second embryo from the base of the uterine horn using a Vernier caliper (150 mm (6") Electronic Digital Vernier Caliper). The appearance of each ovary and the number of corpora lutea present were recorded \[4\].

After the observations rats were sutured and they were injected with a subcutaneous dose of 0.1 mg/kg amoxicillin (Amoxi 15% L.A). Tetraacycline cream (Tetracycline Hydrochloride Ointment USP 3%) was applied on sutures and allowed to recover.

The gestation length was monitored and the number of viable pups, stillborn pups was recorded after delivery. On the postnatal day three, pups were closely examined for the presence of abnormalities as open eyelids, fold pinna and tail abnormalities and their body weights, cranial length, cranial diameter, craniosacral length and the tail length of each pup were measured. Mortality of Pups up to 6 days, the time taken for eye opening, fur appearing and sexual maturation (vaginal opening day) were recorded \[4\].

The following reproductive indices were computed based on the observations made during above procedure;

\begin{align*}
\text{Quantal pregnancy} \% &= \left( \left( \frac{\text{pregnant animals}}{\text{mated animals}} \right) \times 100 \right) \\
\text{Pre-implantation loss} \% &= \left( \left( \frac{\text{corpora lutea} - \text{implants}}{\text{corpora lutea}} \right) \times 100 \right)
\end{align*}

Post-implantation loss \% = \left( \left( \frac{\text{implants} - \text{viable implants}}{\text{implants}} \right) \times 100 \right)

\begin{align*}
\text{Implantation index} \% &= \left( \frac{\text{viable implants}}{\text{corpora lutea}} \right) \times 100
\end{align*}

Litter Index \% = \left( \frac{\text{littered pups}}{\text{implants}} \right) \times 100

\begin{align*}
\text{Live birth Index} \% &= \left( \frac{\text{livel} \text{ly} \text{born} \text{pups}}{\text{littered} \text{pups}} \right) \times 100
\end{align*}

\begin{align*}
\text{Fetal survival ratio} \% &= \left( \frac{\text{surviving} \text{pups}}{\text{viable} \text{implants}} \right) \times 100
\end{align*}

\section*{Acute toxicity evaluation}

Ten mature female rats were randomly assigned in to two groups, each containing five animals. Measuring of body weights and neurotoxicity tests were conducted as pre and post treatment. The ASE of \textit{Macrotlyloma uniflorum} (double the HED: 12.34 g/kg) or distilled water was administered orally using a cannula to the particular group for seven days at the same time 1000-1100h. Overt signs of toxicity, stress, behaviour, food intake and water intake were noted down. Blood was collected on post treatment day via tail bledding under mild ether anesthesia and aseptic conditions. Serum parameters such as SGOT, SGPT (for liver toxicity), urea and creatinine (for renal toxicity) were measured by sending the samples to the laboratory of Norris Clinic, No 49, Norris Canal Road, Colombo. Serum Na* and K* were determined using the atomic absorption spectrophotometer at Department of Chemistry, University of Colombo.

Cage-side examinations of rats used for toxic study were performed daily at 1000-1300h, to detect overt signs of toxicity (salivation, diarrhoea, lacrimation, chewing jaw movements, yellowing of fur, loss of hair), stress (erection of fur and exophthalma), behavioural abnormalities (biting and scratching behaviour, licking of tail, paw, intense grooming behaviour or vocalization) and mortality \[5\].

The food intake of the animals was assessed on 5th day by placing individual rat with 60g of pelleted food for 24 hours. On the next day the weight of the leftover food was measured using a balance (Shimadzu Libr6r Eb-3200h, Shimadzu Corporation, Tokyo, Japan). Food intake was calculated by deducing leftover by the total given and was expressed as g/100g body weight. Likewise, the water intake of the animals was also assessed on 5th day by placing individual rats with 250 mL of water for 24 hours. On the following day the remaining amount of water was measured and water intake was calculated by deducing remaining water by the total given. Water intake was expressed as mL/100g body weight. Initial and final weight of the female rats were obtained using an electronic animal balance (MP 600, Chyo, Japan) to identify possible changes in weight gain.

Strength of the muscles and muscle coordination were measured using bar holding test and Bridge test as described by Plaznik \textit{et al}, 1993 \[6\]. Time taken by each rat to correct its orientation of the body, when it was taken out of its normal upright position (put it on back) was measured using a stop watch as righting reflex \[7\].

\section*{Statistical Analysis}

All data were tabulated and statistically analyzed using SPSS version 20.0. Results were expressed as means ± SEM. Statistical comparisons were made using Mann-Whitney U-test as appropriate. \(P<0.05\) was considered as statistically significant.

\section*{3. Results}

\subsection*{Evaluation of the effect of ASE on early and mid pregnancy}

During early pregnancy, oral administration of the ASE (6.17 g/kg) has significantly \((P<0.05)\) increased the pre-implantation loss in the treated group (45.44 ± 5.42%) compared to the controls (28.96 ± 2.26%) with a significant decrease in implantation index (treated group 54.56 ± 5.42% vs. 71.04 ± 2.26% in control group) as shown in Table 1. Likewise, in mid pregnancy there was a significant reduction in litter weight, tail length, and craniosacral length in the control group compared to the group treated with ASE (Table 2).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Parameter} & \textbf{Control (DW) Group} & & \textbf{Treatment (6.17g ASE/kg) group} & \\
 & \textbf{Early} & \textbf{Mid} & \textbf{Early} & \textbf{Mid} \\
\hline
\textbf{Quantal pregnancy} (%) & 100 & 100 & 100 & 100 \\
\textbf{Pre-implantation loss} (%) & 28.96 ± 2.26 & 27.08 ± 5.14 & 45.44 ± 5.42* & 20.71 ± 3.34 \\
\textbf{Post-implantation loss} (%) & 0.00 & 1.39 ± 1.39 & 1.56 ± 1.56 & 0.00 \\
\textbf{Implantation index} (%) & 71.04 ± 2.26 & 72.92 ± 7.14 & 54.56 ± 5.42* & 79.29 ± 3.34 \\
\textbf{Litter index} (%) & 65.00 ± 35.00 & 72.22 ± 7.34 & 65.23 ± 14.44 & 68.18 ± 18.37 \\
\textbf{Live birth index} (%) & 100.00 ± 0.00 & 91.90 ± 4.23 & 97.14 ± 2.86 & 88.98 ± 5.17 \\
\textbf{Fetal survival ratio} (%) & 65.00 ± 35.00 & 70.83 ± 11.02 & 62.38 ± 12.92 & 59.87 ± 7.77 \\
\hline
\end{tabular}
\caption{Effect of oral administration of the \textit{Macrotlyloma uniflorum} ASE or vehicle on early and mid pregnancy on some reproductive indices}
\end{table}
These events can be mentioned as sperm-oocyte interaction, capacitation or transport through the female genital tract, capacitation or fertilization occurs five to six hours from the mating [11, 12]. To determine the effect on early pregnancy, the ASE of M. uniflorum (6.17 g/kg) was administered orally at 1000-1100 h daily as single doses, subsequent to mating. So, the induction of pre-implantation loss due to the disruption of events associated with fertilization is unlikely. Pre-implantation loss can occur as a result of impairment in the production of growth factors, cytokines, and various types of adhesion molecules by the uterine epithelium at the site of implantation or by the developing blastocyst [13, 14]. Impairment in production of these can induce pre-implantation losses as evident in this study. Non-nutrient bioactive compounds such as phytic acid, flatulence factors, enzyme inhibitors (trypsin, α-amylase) and phenolic compounds (phenolic acid, tannins, flavonoids) present in M. uniflorum seeds [15,17] poses anti-inflammatory, anti-proliferative, vasodilation actions, potential preventive properties against hormonal related cancers [15, 16, 18]. Possibility exists that such a mode of action may have contributed to the observed of pre-implantation loss.

When the blood supply to the uterus is impaired, there is a possibility of enhancement of the pre-implantation loss [19]. Implantation can be inhibited by the lethality to blastocyst [20]. Further, imbalance of oestrogen and progesterone levels could induce pre-implantation losses: critical oestrogen / progesterone ratios are associated with higher pre-implantation and post-implantation loss.

### Table 2: Effect of oral administration of *Macrotymla uniflorum* aqueous seed extract during early and mid pregnancy on some development parameters of pups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (DW) Group Early</th>
<th>Mid</th>
<th>Treatment (6.17g ASE/kg) Group Early</th>
<th>Mid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial length (cm)</td>
<td>1.62 ±0.12</td>
<td>1.67 ±0.07</td>
<td>1.66 ±0.04</td>
<td>1.56 ±0.02</td>
</tr>
<tr>
<td>Cranial diameter (cm)</td>
<td>1.19 ±0.06</td>
<td>1.15 ±0.05</td>
<td>1.13 ±0.04</td>
<td>1.06 ±0.02</td>
</tr>
<tr>
<td>Craniosacral length (cm)</td>
<td>5.70 ±0.07</td>
<td>5.55 ±0.13</td>
<td>5.52 ±0.07</td>
<td>5.19 ±0.06*</td>
</tr>
<tr>
<td>Tail length (cm)</td>
<td>1.90 ±0.08</td>
<td>1.88 ±0.07</td>
<td>1.68 ±0.02</td>
<td>1.59 ±0.02*</td>
</tr>
<tr>
<td>Mean weight of pups (g)</td>
<td>8.16 ±0.79</td>
<td>7.30 ±0.34</td>
<td>6.72 ±0.17</td>
<td>5.96 ±0.37*</td>
</tr>
<tr>
<td>Appearance of fur (days)</td>
<td>3.50 ±0.5</td>
<td>3.33 ±0.33</td>
<td>3.00 ±0.00</td>
<td>3.33 ±0.33</td>
</tr>
<tr>
<td>Opening of eye lids (days)</td>
<td>14.5 ±0.5</td>
<td>15.00 ±0.58</td>
<td>13.20 ±0.20</td>
<td>15.67 ±0.33</td>
</tr>
<tr>
<td>Vaginal opening (days)</td>
<td>31.0 ±1.0</td>
<td>33.00 ±0.57</td>
<td>30.5 ±0.5</td>
<td>32.67±1.453</td>
</tr>
</tbody>
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Values are expressed as Means ± S.E.M.

*P< 0.05 as compared with the control (Mann-Whitney U-test) is considered significant.

### Table 3: Effect of oral administration of *Macrotymla uniflorum* aqueous seed extract on the reaction time of pre-treatment and post-treatment tests

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-treatment (time in seconds)</th>
<th>Post-treatment (time in seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bar test</td>
<td>Bridge test</td>
</tr>
<tr>
<td>Control (DW) group</td>
<td>11.04 ±2.61</td>
<td>44.60 ±9.59</td>
</tr>
<tr>
<td>Treatment (ASE 12.34 g/kg) group</td>
<td>15.28 ±6.29</td>
<td>18.00 ±4.73</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± S.E.M. (n=5)

*P< 0.05 as compared with the control (Mann-Whitney U-test)

### 4. Discussion

The results of this study demonstrated that, oral administration of the *M. uniflorum* ASE (6.17 g/kg) in female pregnant rats during early pregnancy increased the pre-implantation loss and reduced the implantation index compared to the control (DW) group. The evaluation of pre-implantation loss or post-implantation loss can be mentioned as a result of food restriction, stress, or interruption in corpora lutea function (control versus treatment: 6.07 ± 0.64 versus 5.93 ± 0.68 g/100 g body weight), and the average water intake of treated group was significantly (P< 0.05) increased from the control group (DW) (control versus treatment: 10.19 ± 1.18 versus 16.09 ± 0.93 mL/100g body weight). The mean weight gain of two groups was not significantly (P> 0.05) different in rats treated with *M. uniflorum* ASE and DW (control versus treatment: 25.40 ± 1.78 versus 24.60 ± 1.33 mg/dl), creatinine (control versus treatment: 0.62 ± 0.09 versus 0.60 ± 0.03 mg/dl) and serum Na+ (control versus treatment: 4591.8 versus 6395 ppm) and K+ (control versus treatment: 1857.6 versus 1589.6 ppm) levels were not significantly altered, but the level of SGOT (control versus treatment: 46.2 versus 60.8 IU) was significantly increased from the control group (DW) (control versus treatment: 25.40 ± 1.78 versus 24.60 ± 1.33 mg/dl). Further, neuro toxicity was evaluated (Table 3), the latency shown in Bridge test showed a significant (P< 0.05) post-treatment increase in the ASE treated group.

The average food intake was not significantly (P> 0.05) different in rats treated with *M. uniflorum* ASE and DW (control versus treatment: 6.07 ± 0.64 versus 5.93 ± 0.68 g/100 g body weight), and the average water intake of treated group was significantly (P< 0.05) increased from the control group (DW) (control versus treatment: 10.19 ± 1.18 versus 16.09 ± 0.93 mL/100g body weight).

When the general behaviour of the rats is considered, mating occurs between 2200-2400 h and fertilization occurs five to six hours from the mating [11, 12].

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*P< 0.05 as compared with the control (Mann-Whitney U-test)
progesterone ratio is vital for proper implantation [20]. It is recognized that elevated levels of oestrogen and progesterone ratio in the early luteal phase (human) can lead to early pregnancy failure by inhibiting implantation and creating a uterine hormonal milieu which interfere with the embryonic metabolism [22]. Since there is no evidence against or supporting these actions, an exact mechanism for the increased pre-implantation index cannot be pinpointed. According to the results, there was no alteration of post-implantation loss during the experiment. It is known that embryonic toxicity can induce post-implantation losses [23]. Absence of significant increase in death implants and absence of any overt signs of toxicity in treated rats strongly suggest that M. uniflorum ASE is unlikely to have direct toxic effects on the embryos [24]. However, as shown in results, the administration of M. uniflorum ASE (6.17 g/kg) to female pregnant rats during mid pregnancy has resulted in significant decrease in mean body weight, tail length and craniosacral length of the pups. This may indicate intrauterine growth retardation (IUGR) which possibly indicates some embryo toxicity. Since the gestation length was not reduced, it cannot be attributed to the observed IUGR [4]. The IUGR observed in this study could be a result of intrinsic fetal factors such as chromosomal abnormalities or other malformations [4], because of the presence of birth anomalies such as curved tail and fold pinna on the third prenatal day [25, 26]. Since M. uniflorum known to have anti-diabetic properties: the IUGR may be attributed to impaired glucose supply to the fetuses. But, the exact mechanism by which the extract lowered the blood glucose is not known. The spasmolytic action of some chemicals present in herbs reduces the gut motility which may limit the absorption of nutrients into the blood stream [27]. Insufficient nutritional availability in mothers’ circulation may limit the transfer of nutrients via placenta to the fetus, this can be another reason for the IUGR [27, 28]. Since, there is no record on spasmolytic activity of M. uniflorum and suppression in maternal body weight, insufficient nutrition availability unlikely to be a reason for IUGR.

A hormone called rat chorionic mammotropin (rCM) is responsible for accelerating the nutritional transfer through the placenta to the fetus during latter half of the pregnancy [29]. Fetal growth and the weight can be reduced by the impaired production of rCM during gestation days 8 – 15 [29, 30]. Such a mode of action can contribute to the IUGR due to oral administration of the ASE of M. uniflorum during mid pregnancy. Further, one researcher has shown in his study that there is no production of rCM during early pregnancy (gestation days 1-7) [29]. But the exact mechanism of how the ASE of M. uniflorum interferes with the hormone production and secretion is not known. None of the treated rats showed vaginal bleeding. Thus, abortifacient action of the ASE seems unlikely. Further studies should be performed to identify possible causes of pre-implantation loss and intrauterine growth retardation.

The number of corpora lutea and the number of implantation sites are correlated with the implantation proportion [31]. Further, comments that the implantation index is an indirect way to observe the number of ovulations that resulted in fertilized oocytes and in implanted blastocysts. Implantation index also can be taken as an indicator of successful implantation of the blastocyst in the endometrium [32]. According to the results mean number of corpora lutea was not significantly different and there was a significant (P< 0.05) decrease in implantation index of the treated group (6.17 g/kg) compared to control group (DW), it is reasonable to assume that M. uniflorum involved altering the process of blastocyst implantation.

Increased pre-implantation loss and reduced implantation index by treatment of the ASE of M. uniflorum during early pregnancy may indicate that M. uniflorum may impair the reproductive capacity. Since the embryo quality, uterine receptivity, and synchronization of embryonic development and endometrial maturity effect on successful implantation [33], it can be assumed that M. uniflorum ASE may acted directly on the uterus and made inappropriate endometrial environment for implantation which lead to reduction of implantation sites.

Toxicity is considered as the main drawback of indigenous preparations. Most of the time adverse toxic effects can occur due to the inappropriate indications, preparation methods and use of excessive doses or use for a longer period [34]. Otherwise traditional medicine is known to have no side effects [43]. In this study, acute toxicity of M. uniflorum ASE was evaluated following oral administration. The ASE was well tolerated by the female rats showing no overt signs of toxicity, morbidity or mortality.

Parameters as body weight and food consumption are frequently used in toxicity evaluation. Maintaining the weight gain or the body weight of an animal in a relatively constant level is a sensitive, but nonspecific sign of health [35]. According to the result of the present study, the ASE (12.34 g/kg) of M. uniflorum failed to affect the food intake of the animals and weight gain compared to the control group indicating unimpaired health.

Water intake of the treated (12.34 g/kg) group was higher than the control group. This can be due to the stimulation of the thirst cells [36, 37].

In the neurotoxic evaluation of the ASE of M. uniflorum, response time of bar test and righting reflex remained unaltered, but response time in Bridge test was significantly decreased. This result indicates some degree of impairment in muscle coordination without a significant effect on muscle strength [6]. Serum urea, creatinine, potassium, and sodium concentrations are indices of kidney function [38]. Serum urea, creatinine, and potassium concentrations of treated group (12.34 g/kg) were lower but not significantly different from the control group. This indicates that, there was no adverse effect on the kidney function by M. uniflorum ASE (12.34 g/kg). Further, both sodium and potassium ions are important in muscle contraction [39]. Since the alterations in concentrations of electrolyte were not significant, this indicates that, the administering of the ASE (12.34 g/kg) of M. uniflorum did not cause weak muscles or muscle cramps [39]. However, the serum GOT and GPT activities were high in treated (12.34 g/kg) group compared to the control group, but only the SGOT level was significantly increased. SGOT and SGPT are used as biochemical markers in hepatocellular necrosis [40]. SGPT is highly specific to the liver damages while SGOT indicates less specificity to the liver by locating GOT enzyme in the heart, brain, kidney, and skeletal muscle [41]. However, low dose of M. uniflorum seed extract (up to 1 g/kg) is hepatoprotective against paracetamol induced hepatotoxicity in Wistar albino rats [42]. Marked increase in SGOT level observed in this study is likely to be caused by the high dose of the ASE of M. uniflorum administered.

This study shows that consumption of M. uniflorum ASE does not induce overt signs of toxicity, renal toxicity but when
considering hepatotoxicity, long term consumption of *M. uniflorum* ASE daily can induce increase of Serum Glutamic Oxaloacetic Transaminase (SGOT).

5. Conclusion
This study showed that oral exposure of *M. uniflorum* ASE during early gestation of rats is detriment to pregnancy in terms of pre-implantation loss and reduced implantation index. Intake of *M. uniflorum* ASE in mid pregnancy, can cause retardation in pup development. Overall ASE of *M. uniflorum* is well tolerated by the laboratory rats without indications of acute toxicity effects.

6. References