

# The possible mechanisms for the antifertility action of methanolic root extract of *Rumex steudelii*

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## ABSTRACT

**Back ground:** The practice of traditional medicine for the control of fertility in most parts of Ethiopia is based on the uses of plant medicines for many years. *Rumex steudelii* Hochst (*Polygonaceae*), locally known as “Tull” or “Yeberemelas” is one of the traditionally used antifertility plants in Ethiopia. In our previous study, the methanolic extract of *R. steudelii* root was found to show antifertility activity in female rats.

**Objectives:** The present study focused further on the possible mechanisms of the antifertility effect of the methanolic extract of *R. steudelii*.

**Methods:** The effect of the extract on implantation, the uterus weight of immature ovariectomized rats and serum estrogen-progesterone ratio was evaluated. Its effect on isolated guinea pig uterus in the presence and absence of uterine muscle contractions inhibitors was also assessed. Test for *in vivo* abortifacient effect was also carried out.

**Results:** It was found that the extract decreased the number of implantation sites significantly. At a contraceptive dose, it was also observed to have no estrogenic activity in immature rat bioassay. The extract did not affect the serum estrogen-progesterone ratio. It produced concentration dependent increase in uterine muscle contractions similar to those of the standard drug, oxytocin. Incubation of the tissue with three uterine muscle contractions inhibitors revealed that the extract produced uterine contractions perhaps by activating muscarinic and/or histaminic receptors. The *in vivo* abortifacient effect was not seen upon administration of both lower and higher doses of the extract in pregnant rats.

**Conclusion:** All these observations suggest that the extract produced antifertility effect mainly by inhibiting implantation though antiestrogen, progesterone and uterotonic effects could as well be possible mechanisms.

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## INTRODUCTION

*Rumex steudelii* Hochst, whose vernacular name is “Tull” or “Yeberemelas”, is widely distributed in different parts of Ethiopia, such as Tigray, Gondar, Gojam, Wollo, Shewa and Arsi highlands. It is an erect, perennial herb, and grows up to 1m tall<sup>1</sup>. It is traditionally employed in combination along with other medicinal plants to treat various ailments such as hemorrhoids, wounds, eczema, leprosy, tonsillitis<sup>2</sup>. The root of this plant is also used as an abortifacient and in the control of fertility traditionally.

Previous studies on the methanolic extract of the root showed anti-implantation effect in rats<sup>3</sup>. Further investigation of the methanolic extract of the root of this plant reduced significantly the number of litters, prolonged the estrus cycle and the diestrus phase and reduced the wet weight of the ovaries and uterus of Wistar albino rats. The same extract produced antifertility effect in a dose dependent manner and the contraceptive effect manifested a definite period of time<sup>4</sup>. Phytochemical screening of the extract used for the pharmacological tests revealed the presence of saponins, phytosterols and polyphenols as major classes of compounds<sup>4</sup>. Hence, this study was carried out to elucidate the possible mechanism(s) for the antifertility effect of the methanolic extract of the root of *Rumex steudelii* with models not reported previously.

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## MATERIALS AND METHODS

### Materials:

#### Standard drugs:

Powdered estradiol benzoate (Control No.167014), oxytocin (Lot. No. AJP 2AR, Reg. No. 890525A), salbutamol (Lot. No. B070946), diphenhydramine (No.D-3630), and atropine (Batch No. T020701) were obtained from WHO, Choongwae Pharma Corp Seoul, Glaxo Group Ltd Green ford, Sigma Chemical Company and China Ltd, respectively.

#### Collection and identification:

The roots of *Rumex steudelii* were collected around Addis Ababa in December 2002. The plant was identified by a taxonomist and a voucher sample, Herbarium No. RS-2084, was deposited in the Herbarium of Medicinal Plants of Department of Drug Research, Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia.

#### Animals:

All experiments were performed on in-bred adult, cyclic virgin female albino rats (weighing 190-230 g body weight) and guinea pigs (weighing 300-500 g body weight). Immature ovariectomized female rats weighing approximately 100 g were also employed for the study of the estrogenic effect of the extract. All the rats were bred in a standard animal house. Guinea pigs were obtained from Ethiopian Health and Nutrition Research Institute. The rats were housed in polypropylene cages and maintained under environmentally controlled room provided with a 12:12 hrs light and dark cycle for each 24 hrs periods at a temperature of approximately 25 °C. They were fed on a pellets and tap water *ad libitum*. The animals were allowed to acclimatize to the laboratory environment for 1 hr before being subjected to the experiments. All experiments were carried out in a quiet laboratory setting with ambient illumination and temperature close to those of the animal house.

## METHODS:

#### Preparation of extract:

Air-dried and powdered roots of *Rumex steudelii* (300g) were extracted by percolation at room temperature using methanol. The methanolic extract was concentrated under vacuum in a rotary evaporator to yield dark brown semisolid mass which is further dried under vacuum oven drier (at approximately 25°C for 3 days) to give (as a percentage of dried powdered plant materials) 8.5%

solid residue. This extract was reconstituted in distilled water to get the desired concentration for all pharmacological tests.

#### Test material administration:

Administration of the extract was done with intragastric tube on the basis of the animal's body weight. The dose for each animal was calculated considering the previous study [3] and the dose used in antifertility study on the same part of the plant in our previous studies<sup>4</sup>.

#### Study on anti-implantation activity:

The anti-implantation activity<sup>5</sup> was determined according to the method of Mukherjee<sup>5</sup>. Ten mature females colony bred Wistar albino rats were divided into two groups (5 female rats per group). One group was used as a control and the other group was used as a test group. Both groups were left over night with males (in the ratio of 2 females to 1 male) and the vaginal smear was examined for motile spermatozoa in the morning. The day on which the spermatozoa were found in the smear was considered the first day of pregnancy (Day 1). A 2.2g per Kg of body weight of the extract was administrated intragastrically for 10 days from day 1 to day 10 of pregnancy for the test group and same volume of vehicle for the control group. On day 11, both groups of rats were laparotomized under light ether anesthesia to determine the number of implantation sites in the horns of the uteri. The presence of significant difference in the mean number of implantation sites between the extract and the control was taken as a positive response.

#### Determination of Estrogenicity:

Fifteen colony breed immature Wistar strain albino female rats (average body weight of 100 g) were bilaterally ovariectomized under light ether anesthesia according to the method described by Vogel [6]. Fifteen days after the ovariectomy, the animals were grouped in three groups (5 animals/ group).

The first group was treated with test extract (2.2g per Kg of body weight daily) for 7 days by intragastric gavages. The 2<sup>nd</sup> group received estradiol benzoate in olive oil (0.1mg per kg of body weight daily) subcutaneously for 7 days and kept as positive control. The last group was treated with the vehicle for 7 days by intragastric gavages, and was employed as negative control.

On the 8<sup>th</sup> day, all the animals were sacrificed, and their uteri were dissected out, freed from surrounding tissues, blotted on filter paper and weighed quickly on a balance sensitive to 0.0001 g. The uterine ratio was calculated by dividing uterine weight in milligrams by body weight in grams. The increase in the uterine ratio was

associated with the estrogenic effect of the extract as described by Vogel<sup>6</sup>.

**Effect on serum estrogen and progesterone:**

In this experiment the test group (5 female rats/group) was treated with 2.2 g/ Kg for 10 days by intragastric gavages. The control group (6 female rats/ group) was treated with vehicle in the same way as the test group. On the 11<sup>th</sup> day, both the control and the test group were anesthetized and blood was drawn by cardiac puncture. The blood was allowed to coagulate for an hour. The separation of the serum from other cellular components of the blood was done by centrifuging the coagulated blood at 3800-rev/ min. for 10 minutes. The sera were collected and stored in deep freezer (-20 °C). After two weeks the sera were analyzed for estrogen and progesterone by the methods of electrochemical luminescence immuno assay (ECLISA, Elecsys® systems1010/2010/MODULA ANALYTICS E 170) using human kits.

**In vitro assay for uterotonic activity:**

Unprimed virgin guinea pigs (300-500 g) were sacrificed by a heavy blow to the head, and the two horns of the uterus were dissected out and freed from surrounding tissues. Each horn was then mounted in an organ bath containing De Jalon's Solution with the following composition (mmol) NaCl 154.0, KCl 5.6, CaCl<sub>2</sub> 0.5, NaHCO<sub>3</sub> 6.0 and glucose 2.8. This solution was constantly aerated with an aerator. The bath temperature was adjusted between 32-34 °c to reduce spontaneous uterine contractions. The whole preparation was allowed to equilibrate for 45 minutes according to the method described by Calixto *et al* [7]. The contractile activity was then measured using an isometric force transducer (Grass Model 7E Polygraph, USA).

Isotonic contractions of the uterine muscle with different extract concentrations were recorded, and concentration- response curves were constructed. The extract additions were cumulative. In the same way, the concentration- response curve of the standard drug, oxytocin was constructed. The effect of the extract after thorough washing of the preparation was observed and recorded.

The effect of the extract was also investigated in the presence of three uterine muscle contractions inhibitors (35 ig/ml atropine, 1.17 mg/ml diphenhydramine and 7.14 ig/ml salbutamol), which were equilibrated with the tissue for 45 minutes. Extract concentration- response curves were

constructed after the tissues were incubated with above mentioned uterine muscle contraction inhibitors.

The EC<sub>50</sub> value for the extract, i.e. the concentration causing half maximal contraction was determined.

The intrinsic activity of the extract was calculated by using the following formula<sup>8</sup>.

**Abortifacient activity:**

Colony bred adult Wistar female rats (200-210 g of body weight) were allowed mating with males of proven fertility (by keeping the animal in one cage in the ratio of two females to one male). Vaginal smears were examined every morning for the presence of spermatozoa. The day when the spermatozoa were present in the vaginal smears was considered day 1 of pregnancy. These rats were then divided in to 3 groups (6 rats/ group). The first and the second groups received the root extract by intragastric gavage, 3.0g/Kg and 6.0g/Kg of body weight, respectively, the third group received the vehicle on day 15 of pregnancy. All animals were observed daily, and autopsied 48 hrs after dosing. A significant change in the number of viable fetuses was considered a positive response according to the method described by Oshima *et al*<sup>9</sup>.

**Statistical analysis:**

The data were statistically analyzed using statistical packages (GraphPad Instat) and the level of significance was tested with unpaired t-test. Data were expressed as mean ± standard error of the mean.

**Results**

**Study on anti- implantation activity:**

The methanolic extract of *R. stuedelii* root was found to reduce significantly (P= 0.033) the number of implantation sites from 11.2 ± 0.80 to 8.8 ± 0.80

**Determination of Estrogenicity:**

Figure 1 depicts the estrogenic effect of *R. stuedelii* when tested in immature ovariectomized rats. In the control animal the uterus presented a typical infantile condition. The administration of estradiol benzoate provoked significant increase(P=0.016) in the uterine wet weight. The administration of methanolic extract of *R. stuedelii* at 2.2g / Kg of body weight for 10 days did not increase uterine wet weight (P= 0.394).

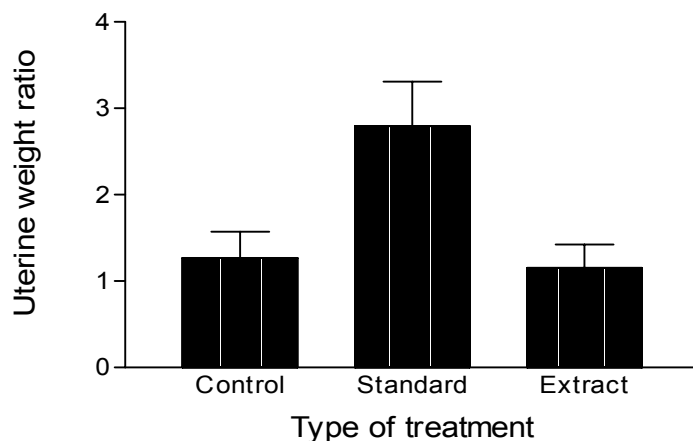


Figure1. The effect of *R. steudelii* root methanolic extract in comparison with that of the standard drug (estradiol benzoate) and vehicle treated on the wet weight of immatured ovariectomized rat uterus.

### Effect on serum estrogen and progesterone:

The result indicates that there was a slight decrease in the serum estrogen level, an increase in serum progesterone level and an increased serum estrogen-progesterone ratio (table 1) though it was not significant (P value 0.406, 0.430 and 0.320 respectively).

**Table 1: Effect of *R. steudelii* root methanolic extract (2.2g/Kg/day for 10 days) on serum estrogen & progesterone in rats**

	Estrogen(pg/ml)	Progesterone(pg/ml)	Estrogen-progesterone ratio
Control (n=6)	27.68 ± 8.50	14.28 ± 2.88	2.49 ± 1.19
Extract (n=5)	24.90 ± 7.40	15.39 ± 5.84	3.61 ± 2.07

Data are Mean ± SEM

### Uterotonic activity:

Initial experiments with the methanolic root extract of *R. steudelii* produced a concentration dependent increase in tension of the tissue with EC<sub>50</sub> value of 0.32 mg/ml which was obtained by interpolation in Figure 2. Where as the EC<sub>50</sub> value of oxytocin was 1.99X10<sup>-6</sup>mg/ml. The intrinsic activity of the extract was approximately equal to one, which was obtained by taking the ratio between the maximum effect of the extract and that of oxytocin i.e. 99.4%/100%.

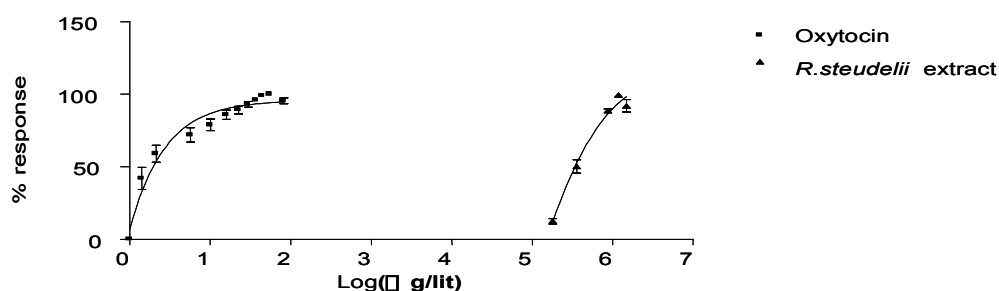


Figure 2 :Contractile response of guinea pig uterus to oxytocin and the extract. Data are expressed as means ± SEM, n =6 .

When the tissue's muscarinic receptor was blocked with an antagonist (atropine), however, the concentration-response curve of the extract was shifted dextrally with a decrease in maximal tension (*Figure 3*).

In a similar but more potent manner,  $\alpha_2$  receptor agonist (salbutamol, 7.14 $\mu$ g/ml) also caused right-ward displacement of the concentration- response curve (*Figure 3*). The same figure shows that blocking histaminic receptor by diphenhydramine (1.17mg/ml) resulted in total abolishment of the contractions of the tissue at all concentration of the extract added.

All the effects produced by the extracts on the isolated uterine muscles were not reversed by intermittent washings of the preparation with physiological solution over 10 minutes.

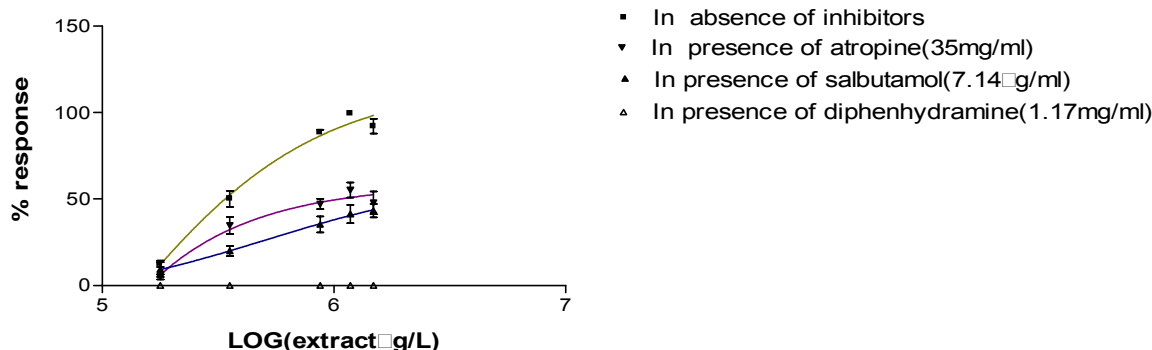


Figure 3: Antagonism of extract-induced contraction of guinea pig isolated uterus by atropine, salbutamol and diphenhydramine. Concentration- response curves to *R. steudelii* extract were constructed in the absence or presence of inhibitors which was equilibrated in the tissue for 45 minutes prior to extract stimulation. Data are shown % maximum extract -induced contraction prior to incubation with antagonist and are expressed as means  $\pm$  S.E.M. of atleast six independent experiment.

#### Abortifacient activity:

In this work the number of viable fetus was used as an index for the presence of abortifacient effect. Treating the pregnant rats with the extract did not show a significant change in the number of the viable fetus (*table 2*) with both smaller (3.0g/Kg) and higher doses (6.0g/Kg) (P value 0.051 and 0.095 respectively).

**Table 2: Abortifacient effect of a single administration of methanolic extract of the root of *R. steudelii* in pregnant rats**

Dose(mg/100g)	N	Viable fetus
Control	5	8.4 $\pm$ 0.400
300	6	6.5 $\pm$ 0.885
600	6	7.0 $\pm$ 0.836

Data are Mean  $\pm$  SEM

#### DISCUSSION

None of the previous studies documented the possible mechanism for the antifertility effect of the *R. steudelii* extract, only anti-implantation effect was reported by Desta<sup>3</sup>.

The antifertility effect of the methanolic extract of *R. steudelii* might be attributed to one or more mechanisms. One possible mechanism could be inhibition of implantation, which was shown by

the significant decrease in the implantation sites. The anti-implantation effect of this extract may due to the disturbance of endocrine-endometrial synchrony which is dependent on estrogen and progesterone balance. Factors other than the hormones such as histamine<sup>10</sup>, prostaglandins<sup>11</sup>, proteolytic enzymes<sup>10</sup>, NOS<sup>12</sup>, alkaline phosphatase<sup>13</sup>, interleukins<sup>14</sup>, and Leukemia inhibitory factors<sup>15</sup>, which are important for implantation, may also be affected by the extract. However, further studies need to

be carried out to confirm whether the extract could interfere with the above mentioned mediators.

The difference between the number of implantation sites from the present study and that of the number of litters from the previous study<sup>4</sup> at the same dose could suggest the presence of resorption of embryos as previously reported by Ghosh<sup>8</sup>.

Large numbers of antifertility plant extracts are known to exhibit estrogenic activity in rats<sup>16</sup>. Estrogenic substance may cause the expulsion of ova from the tube, disruption of luteotrophic activity of the blastocyst, disrupt the functional equilibrium between the endogenous estrogen and progesterone which may result in failure in fertility. Increasing in the wet weight of uterus of substance treated ovariectomized immature rats may indicate that the substance has estrogenic effect<sup>5</sup>. In the present study, however, methanolic root extract of *R. stuedelii* did not show an increase in the uterine wet weight of immature ovariectomized rats, which indicates the extract, did not have a direct estrogenic property.

The hypothalamus has threshold requirement for estrogen to cause a massive release of LH by the pituitary gland. This surge of LH is the trigger which initiates the rupture of the follicle (ovulation)<sup>17</sup>. Furthermore, studies showed that administration of antiestrogen or a decrease in the serum level of estrogen produces a breakdown of embryo-endometrial maturation and their synchronization for implantation<sup>18</sup>. The decrease in the serum estrogen level and a change in the estrogen to progesterone ratio that was observed in the current study though it was not significant may have physiological importance. This is because the threshold requirement for estrogen may not be necessarily statistically significant. Hence, it is possible to consider the decrease in the serum estrogen level as one of the mechanisms by which the extract produced antifertility effect.

Generally, it is known that an increase in the serum progesterone level prevents pregnancy through inhibition of ovulation and alteration of cervical mucus. In the present study it was observed that there was slight increase in the serum level of progesterone though it was not significant. Therefore, by analogy the slight increase to progesterone level might also be another mechanism for the antifertility effect of *R. stuedelii* extract.

The observed uterotonic response by the extract was characterized by an increase in the magnitude and frequency of uterine contractions

indicating the abortifacient effect of the extract, which might be another mechanism. The finding from the isolated uterine muscle studies also showed that the extract produced an equal maximal effect on uterine muscle contraction to that of the standard drug, oxytocin, though less potent. The persistent contraction observed after the washing out of the tissue may indicate that the extract bind to its receptors irreversibly.

The decrease in the magnitude of the maximal effect upon incubating the tissue with muscarinic antagonist (atropine) indicates that the effect of the extract on the tissue may be mediated through activation of the muscarinic receptor. While the decrease in maximal effect of the extract upon incubating the tissue with  $\hat{a}_2$  agonist (salbutamol) suggests that  $\hat{a}_2$  receptors may not be involved in the extract induced contraction.

Total abolition of the extract induced contractions by histaminic receptor antagonist (diphenhydramine) indicates that the extract may enhance uterine muscle contraction mainly by stimulation of the histaminic receptor or activation of muscarinic and histaminic receptors together as the drug is a nonselective blocker of both receptors.

The *in vitro* uterotonic finding on isolated guinea pig uterus did not correlate with the *in vivo* abortifacient effect in pregnant rats in the present study at any of the dose of the extract studied. This is may be due to various reasons such as species differences and pharmacokinetic variables in the *in vivo* studies.

## CONCLUSION

The present study suggests inhibition of implantation reduction of estrogen level and increment of progesterone level as the possible mechanism of antifertility effect of the methanolic extract of *R. stuedelii*. The increase in uterine muscle contraction through activation of muscarinic and/or histaminic receptors might also be another possible mechanism though it did not correlate with the *in vivo* studies in other species.

## RECOMMENDATION

Further work is necessary to identify active component(s) responsible for the observed effect and the exact mechanism of action. Work on the semi purified total extract is in progress to validate the present findings.

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