

ANTIFERTILITY EFFECTS OF *POUZOLZIA MIXTA* IN FEMALE WISTAR RATS

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*E-mail: crusike@wsu.ac.za**Abstract**

The continued use of plants by women to prevent pregnancy suggests there are plants out there with potential use as contraceptives. In Zimbabwe, *Pouzolzia mixta* is used as a "morning after" contraceptive, thus it may possess postcoital antifertility activity. To test contraceptive activity, animals (n=8/group) were orally pretreated with aqueous (AqPM) or ethanolic (EtPM) extract of *P. mixta* at 300mg/kg b.wt for 7 days followed by mating with continued treatment for 10 days post-conception. To test for postcoital activity, treatment was initiated on day-1 of pregnancy and continued for 10 days. Laparotomy was performed and implantations counted. For estrogenic activity, immature ovariectomised rats were treated for 7 days after which vaginal opening and uterine weights were determined. In vitro oxytocic effects were performed using uterine tissue in an organ bath with De Jalon's solution. Acetylcholine (Ach) was the positive control. Results showed modest contraceptive activity with EtPM more effective in inhibiting fertility compared to AqPM (37.5% vs 25%) with a similar trend for antiimplantation effects (31% vs 19%). There was potent postcoital antifertility effects with AqPM more effective in inhibiting implantation (94.6% vs 86%) and fertility (87.5% vs 75%) compared to EtPM. Immature rat bioassay for estrogenic activity demonstrated pronounced estrogenic activity by both extracts. Oxytocic effects at 400ng/ml were more pronounced for the AqPM (92% of 100ng/ml Ach) than EtPM (25% of 100ng/ml Ach). Findings demonstrate the antifertility effects of aqueous and ethanolic extracts of *P. mixta*. The antifertility effects may be attributed to antiimplantation, estrogenic and oxytocic effects of the plant extracts.

Key words: *Pouzolzia mixta*, antifertility, antiimplantation, estrogenic activity, oxytocic

Introduction

The use of plants for medicinal, mythical and fertility regulating purposes has been practised in African societies for centuries. However, because fertility was very highly regarded and associated with the preservation of the tribe, contraception was not openly discussed. Like most traditional societies, large families were especially prized (Bourdy and Walter, 1992). However, contraception was practiced and some plant contraceptives continue to be used today. In the ongoing search for more convenient and effective products to increase reproductive choice, there is a need to explore methods that utilise natural products (Chiuriri, 2000). The perennial shrub *Pouzolzia mixta* (*hypoleuca*) with local names: soap brush (English), isikhukhukhu (Ndebele) or munanzva (Shona), belongs to the Urticaceae family. It is claimed to possess antifertility activity. Its native distribution extends from northern South Africa, Botswana, Zimbabwe, and then further north as far as Malawi (Palgrave and Palmer 2002; Palmer and Pittman, 1972). The root is taken orally by women as a powder in porridge and as an infusion or decoction the morning after sexual intercourse. Therefore this plant may possess postcoital anticeptive activity (Sewani-Rusike, 2010). *Pouzolzia mixta* is also used in Southern Africa by women for treating painful uterus, for expulsion of retained placenta, to treat infertility in women, to treat venereal diseases and to dilate the birth canal (Gelfand *et al.*, 1985). Indeed, despite the availability of synthetic contraceptive medications, alternatives are still needed that may have less side effects and to increase the contraceptive supermarket (Sheeja *et al.*, 2009; Vaidya *et al.*, 2006). Several other plants used in different cultures for contraception and experimentally demonstrated to possess antifertility activity include: *Cissampelo pareira* (Ganguly *et al.* 2007), *Wrightia tinctoria* (Keshri *et al.*, 2008), *Hibiscus rosa-senensis* (Vasudeva and Sharma, 2008 a and b), *Spondias mombin* (Chukwuka and Isek, 2008), *Crataeva nurval* (Bhaskar *et al.*, 2009), *Plumbago rosea* (Sheeja *et al.*, 2009), *Indigofera linnaie* (Pradeepa *et al.*, 2012) and *Tabernaemontana divaricata* (Mukhran *et al.*, 2012). The antifertility effects of *P. mixta* root have not been scientifically validated. The aim of this study therefore was to investigate antifertility activity of crude extracts of *P. mixta* in female rats.

Materials and Methods**Plant material**

The *Pouzolzia mixta* roots used in this study were collected from Hwedza in the Mashonaland East region of Zimbabwe.

<http://dx.doi.org/10.4314/ajtcam.v10i3.21>

Mr Mavi a botanist with the National Herbarium of Zimbabwe collected and authenticated the plant material. The roots were shade dried and pulverised by grinding using a cutter mill. The pulverized material was passed through a sieve to obtain a powder.

Preparation of extracts

Aqueous and ethanolic extracts of *P. mixta* were prepared by mixing 500 g of plant powder with 1000 ml of either deionised water or 99.8% ethanol and agitated overnight at room temperature (Labcon 508U shaking incubator). Three consecutive extractions were made and filtered using Whatman No. 1 filter paper. All filtrates were pooled. The aqueous filtrate was freeze dried to give a powder (Modulyo Edwards). Yield was 13.1%. The ethanolic filtrate was dried in a rotary evaporator and yield was 9.2%. For animal studies, aqueous extract was reconstituted in normal saline and ethanolic extract in normal saline with 0.01% Tween 20.

Preliminary phytochemical screening

Qualitative phytochemical studies were performed on both aqueous (AqPM) and ethanolic (EtPM) extracts for flavonoids and tannins using ferric chloride test (Martin and Martin, 1982), alkaloids, proteins, tannins and glycosides using standard methods (Harborne, 1984; Longanga et al, 2000).

Animals

Adult female Sprague Dawley rats (180-250g) showing normal regular oestrus cycle as confirmed by daily vaginal smears were selected for studies. Male rats of proven fertility were used for mating studies. Albino mice of both sexes were used for acute toxicity studies. All animals were housed in polypropylene cages and maintained on a 12h light:12hr dark cycle, controlled temperature of 24-28°C, with water and solid pellet food (National Foods, Zimbabwe) *ad libitum*. Animal studies were approved by the Institutional Animal Ethics Committee.

Acute toxicity

The method described by Turner (1971) was used, with minor modifications. Adult mice of either sex were assigned into five groups of five mice each. The different groups received orally: saline (control), 2000 and 4000mg/kg b.wt of either AqPM or EtPM after fasting overnight. Animals were observed over 72 hours for behavioural changes and mortality.

Contraceptive activity

The method described by Williamson et al (1996) and Tafesse et al (2005) was used. A total of 24 female rats, irrespective of stage of oestrus cycle, were divided into 3 experimental groups of 8 animals/group. The rat treatment groups were as follows: Group 1- Control (CON = normal saline orally); Group 2- 300 mg/kg body weight *P. mixta* aqueous extract (AqPM); Group 3- 300 mg/kg body weight *P. mixta* ethanolic extract orally (EtPM). The animals were treated for seven days then allowed to cohabit with males of proven fertility at a ratio of 1male:2 females on the 8th day. Animals were examined the following morning for evidence of successful copulation. Animals with spermatozoa in vaginal smears or mucus plug were separated from male partners and this was considered day one of gestation (Desta, 1994). The mated animals were distributed into the treatment groups. Animal treatments were continued until day 10 of pregnancy. On the 10th day, female rats were euthanized by deep sodium pentobarbital (65mg/kg IP; Sigma) anaesthesia. A caesarean dissection was performed on each female rat to expose the uterine horns. The uteri were examined for pregnancy status and number of implantation sites. Mean number of implantation were compared to controls. Mean number of implantations was calculated as total implantation sites divided by the number of pregnant rats in the group. Antiimplantation and antifertility activity was calculated as:

$$\text{Antiimplantation activity \%} = \frac{\text{No. of implants in control} - \text{No. of implants in test group}}{\text{No. of implants in control group}} \times 100$$

$$\text{Antifertility activity \%} = \frac{\text{No. of non-pregnant animals}}{\text{Total number of animals}} \times 100$$

Postcoital antifertility activity

The method described by Vasudeva and Sharma (2008 a and b) was used. Animals with regular oestrus cycle were mated as described above without pre-treatment with extract. Animals with confirmed successful copulation were separated from the male and treatment with saline (CON) or *P. mixta* (AqPM and EtPM) at 300mg/kg was commenced on day-1 of pregnancy for 10 days. On the 10th day, female animals were euthanized and dissected to examine pregnancy status and implantation sites. Antiimplantation and antifertility activity (%) was calculated.

<http://dx.doi.org/10.4314/ajtcam.v10i3.21>

Estrogenic activity

Uterine weight and vaginal opening were used to assess estrogenic activity as described previously (Vasudeva and Sharma, 2006, 2007). All surgical procedures were performed after intraperitoneal (i.p.) injection of 75mg/kg ketamine hydrochloride and 8mg/kg xylazine anaesthesia. Immature female Sprague Dawley rats 10-13 days of age were ovariectomised (Estai et al., 2011). After sterilising the ventral surface of the abdomen with alcohol and iodine solution, a midline incision was made to access the peritoneal cavity. After ligation of the fallopian tubes, ovaries were removed. The incision was sutured using sterile nylon thread. Animals were allowed to recover for 10 days with appropriate care for aseptic healing of the wound. At age 20-23 day, ovariectomised rats were divided into 4 groups of 6 animals each consisting of CON (saline), AqPM (300mg/kg), EtPM (300mg/kg) and conjugated equine oestrogen (CEE, 0.2mg/kg,) as positive control. All animals were treated orally by gavage for 7 days. On the 8th day, animals were sacrificed by anaesthesia overdose and vaginal opening inspected and recorded. Uteri were dissected out, blotted on absorptive paper and weights recorded.

Effect of extract on isolated uterine tissue

The method described by Tafesse et al (2005) was adopted with minor modifications. Non pregnant rats were used for the uterine preparation study. Animals were killed by stunning and exsanguination. The abdomen was opened and uterine horns quickly removed and placed in a dish with warmed De Jalon's solution (composition in mM NaCl-154, KCl-5.63, CaCl₂ -0.648, NaHCO₃ -5.95 and glucose-2.77). 3cm uterine strips were prepared and mounted in 30ml organ bath connected to Grass Polygraph Model 07 under 1gram resting tension. The organ bath was thermostatically maintained at 37°C with continuous aeration with carbogen (95% O₂ + 5% CO₂). Tissues were allowed to equilibrate for 45 min before they were challenged with *P. mixta* or acetylcholine, the reference drug. An acetylcholine (ACh) dose response curve was constructed using 25ng/ml, 50ng/ml, 100ng/ml and 200ng/ml doses. ACh at 100 ng/ml was selected as the dose producing maximal contraction of the uterine tissues (100% contraction). AqPM and EtPM extracts were tested at final bath concentrations of 50, 100, 200 and 400ng/ml. For each treatment, the drug was left in contact with tissues for 3 minutes and then washed with De Jalon's solution before the next dose could be added. The effect on uterine tissue for each sample was calculated as % of reference maximal contraction (ACh 100ng/ml). Six uterine strip preparations were used for each sample and the means were calculated.

Data analysis

All experimental data were expressed as mean \pm standard error of mean (SEM). Statistical comparison was performed using GraphPad InStat software version 3 using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. Values were considered significantly different when $P < 0.05$.

Results

Preliminary phytochemical screening

Qualitative phytochemical analysis of the extracts revealed the presence of flavonoids, polypeptides, tannins and glycosides in both extracts. Alkaloids and carbohydrates were not detected.

Acute toxicity

No behavioral changes and no mortalities were observed in all control and treated (AqPM and EtPM) mice at both doses of 2000 and 4000 mg/kg b.wt.

Contraceptive activity

Oral treatment with extract for 7 days before and for 10 days after confirmation of mating resulted in both aqueous and ethanolic *P. mixta* extracts at 300mg/kg b.wt inhibiting implantations in female rats ($P < 0.05$ and $P < 0.01$ respectively). Both extracts exhibited contraceptive effects as demonstrated by antiimplantation and antifertility activity. However, EtPM was more effective in inhibiting implantation (31% vs 19%) and fertility (37.5% vs 25%) compared to AqPM (Table 1).

Postcoital antifertility activity

Oral treatment with extract for 10 days after confirmation of mating resulted in both aqueous and ethanolic *P. mixta* extracts at 300mg/kg b.wt inhibiting implantations in female rats ($P < 0.001$). Although both extracts exhibited massive postcoital activity as demonstrated by antiimplantation and antifertility effects; AqPM was more effective in inhibiting implantation (94.6% vs 86%) and fertility (87.5% vs 75%) compared to EtPM (Table 2).

<http://dx.doi.org/10.4314/ajtcam.v10i3.21>

Table 1: Contraceptive activity of *P. mixta* aqueous (AqPM) and ethanolic (EtPM) extracts. Animals were treated orally for 7 days prior to mating and continued for 10 days after confirmation of mating.

Treatment Groups	No. of pregnant rats	Mean implantations	Antiimplantation activity (%)	Antifertility activity (%)
Control	8	9.63 ± 0.5	0	0
AqPM (300mg/kg)	6	7.88 ± 0.35*	19	25
EtPM (300mg/kg)	5	6.63 ± 0.26**	31	37.5

Mean implantation values are expressed as Mean ± SEM (n=8/group)

*P<0.5; **P<0.01 compared to control group.

Table 2: Postcoital antifertility activity of *P. mixta* aqueous (AqPM) and ethanolic (EtPM) extracts. Animals were treated orally for 10 days after confirmation of mating.

Treatment Groups	No. of pregnant rats	Mean implantations	Antiimplantation activity (%)	Antifertility activity (%)
Control	8	9.25 ± 0.25	0	0
AqPM (300mg/kg)	1	0.5 ± 0.5***	94.6	87.5
EtPM (300mg/kg)	2	1.25 ± 0.84***	86	75

Mean implantation values are expressed as Mean ± SEM (n=8/group)

***P<0.001 compared to control group.

Estrogenic activity

Oral treatment with *P. mixta* extracts (AqPM and EtPM) at 300mg/kg b.wt caused a significant increase (P<0.001 vs control) in uterine weights in immature rats. However, uteri from EtPM treated immature rats showed a greater increase in weight (P<0.001) compared to AqPM treated rats. Thus, EtPM exhibited greater estrogenic activity compared to AqPM. However, both extracts resulted in earlier opening of vaginas compared to controls (Table 3).

Table 3. Oestrogenic activity of *P. mixta* aqueous (AqPM) and ethanolic (EtPM) extracts in ovariectomised immature rats. Conjugated equine oestrogen (CEE) was positive control. Animals were treated for 7 days.

Treatment Groups	Uterine weight (mg)	Vaginal status
Control	45.43 ± 1.49	closed
CEE	143.2 ± 1.74***	open
AqPM	72.18 ± 2.0*** ^a	open
EtPM	96.68 ± 2.03*** ^a	open

Mean uterine weights are expressed as Mean ± SEM (n=6/group)

***P<0.001 compared to control group.

^aP<0.001 AqPM compared to EtPM

In vitro uterine activity

Sequential additions of aqueous and ethanolic extracts of *P. mixta* at 50-400 ng/ml significantly increased (P<0.001) uterine contractions in vitro in a dose dependant manner. AqPM was more effective in increasing uterine contractions with the highest dose producing contraction comparable to 100ng/ml Ach (92% vs 100%). EtPM only produced modest contractions with 25% contraction at 400ng/ml compared to 100% produced by 100ng/ml Ach (Figure 1).

Discussion

The continued use of plants by women to prevent pregnancy suggests that there are plants out there with potential use as contraceptives. Therefore studying such plants is a good start towards discovering contraceptives to add to the limited contraceptive supermarket for women. *P. mixta* is used as a "morning after" remedy by women of Zimbabwe to prevent pregnancy (Sewani-Rusike 2010).

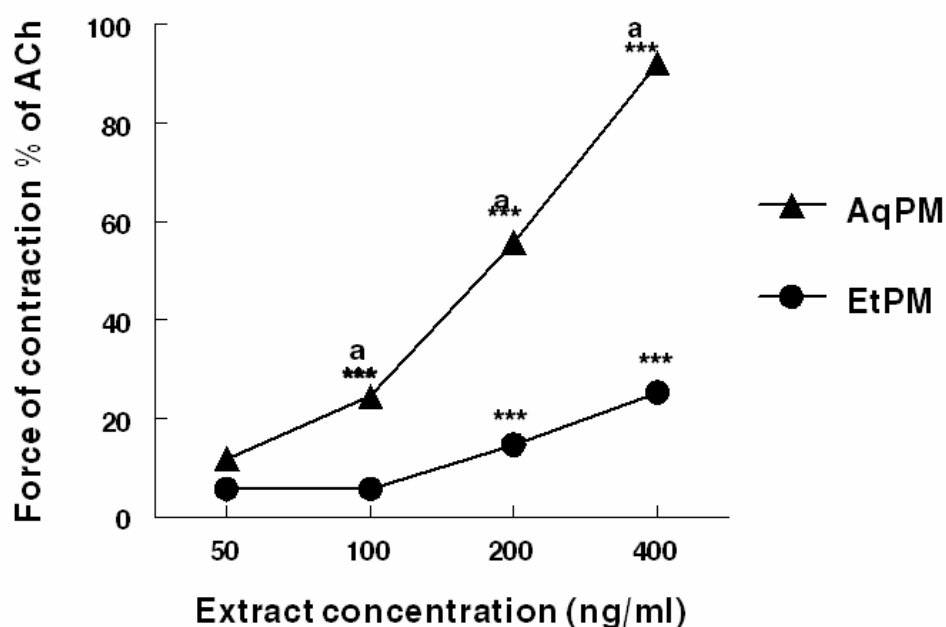


Figure 1. Percentage contraction compared to ACh at 100ng/ml (100%) recorded at different tissue bath concentrations of aqueous (AqPM) and ethanolic (EtPM) extracts of *P. mixta*.

***P<0.001 next higher dose compared to lower dose of same extract.

^aP<0.001 AqPM compared to EtPM

Preliminary phytochemical analysis of this plant showed presence of flavonoids, polypeptides, tannins and glycosides with undetected alkaloids and carbohydrates. The plant was safe in rodents up to a dose of 2000mg/kg b.wt as determined by acute toxicity studies. Results of the present study demonstrated that *P. mixta* possesses primarily postcoital, estrogenic and oxytocic activity with modest contraceptive activity.

In order for implantation to occur, especially in the rat, the exact equilibrium of estrogen and progesterone hormones must be attained to create a milieu ideal for implantation (Psychoyos and Prapas, 1987). Thus, *P. mixta* showed contraceptive activity with inhibition of implantation and fertility rates, ethanolic extract being more effective than the aqueous extract (Table 1). Estrogenic activity is present in a variety of steroidal and nonsteroidal compounds. Nonsteroidal compounds with steroidal activity include phytochemicals such as flavonoids, alkaloids and phenolics (Anderson et al., 1972; Srivastava et al., 2001; Kushalani et al, 2006). The steroidal activity demonstrated in our study may be due to flavonoids detected in the preliminary phytochemical study. And indeed, the nonpolar ethanolic extract demonstrated a higher estrogenic activity than the polar water extract. Where previous studies have demonstrated high contraceptive activity associated with estrogenic activity (Srivastava et al., 2001; Kushalani et al, 2006, Hyacinth et al, 2011) our studies demonstrated modest contraception despite the potent estrogenic activity. Instead, we have demonstrated a highly effective postcoital and oxytocic activity.

Postcoital activity is the mechanism used in emergency contraception to prevent pregnancy after unprotected sexual intercourse. Indeed, in agreement with our study, previous studies have demonstrated an association between postcoital activity with potent estrogenic and oxytocic activities (Uguru et al., 1998; Ayinde et al, 2006; Keshri et al, 2008, Hyacinth et al, 2011). The rat endometrium is sensitive to blastocyst signals in the morning of day 5 (Singh and Kamboj, 1992), but can experience failure of blastocyst implantation due to hostile uterine environment or hypermotility (Hafez and Hafez, 2000; Emiliani et al, 2005). Hypermotility may result in accelerated movement resulting in early arrival of the embryo to a non receptive uterus, and failure of the blastocysts to implant (Cummings and Perreault, 1990). We propose that *P. mixta* estrogenic activity created unfavourable endometrial microenvironment while the oxytocic activity caused hyper motility of the myometrium also preventing implantation. According to some investigators, prolonged phytoestrogen therapy might present with health hazards similar to classic oestrogens (Chan et al, 2002). With use of *P. mixta* in emergency contraception, exposure to phytoestrogens would not be prolonged and therefore deleterious effects of prolonged use may not be an issue.

<http://dx.doi.org/10.4314/ajtcam.v10i3.21>

We conclude that *P. mixta* has potential as an emergency contraceptive due to the demonstrated postcoital activity whose mechanism could be attributed to antiimplantation, estrogenic and oxytocic activity. The potential of this medicinal plant as a useful source of antifertility agent warrants further investigation.

Acknowledgements

This work was supported by the Michael Gelfand Research Foundation of Zimbabwe. The author expresses gratitude to the foundation and the Board of Trustees.

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