EVALUATION OF SPERMICIDAL ACTIVITY ON ROOT METHANOLIC EXTRACT N-BUTANOL FRACTION OF ANDROGRAPHIS PANICULATA (FAMILY- ACANTHACEAE)

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ABSTRACT
Andrographis paniculata (Burm.f.) Wall. ex Nees., (Family- Acanthaceae) (English name-King of Bitters, Tamil name-Nilavempu) is an annual herbaceous plant and is extensively cultivated in Southern Asia, China and some parts of Europe. The aim of this study is to highlight the work on plant drugs and their bioactive extracts involved in male anti-fertility mechanism. The aqueous extract of Andrographis paniculata. In the present study we have evaluated the effective spermicidal concentration of this extract on male albino rat sperm, by conducting “Sander-Cramer test”. The minimum effective spermicidal concentrations of Andrographis paniculata root extract butanol fraction was found to be 12±0.26 mg/ million sperm. After there were no morphological changes observed in the head, mid –piece & tail of sperm. In the in-vivo study, a dose dependent reduction in the epididymal sperm count and percentage motility were observed. These results showed that Andrographis paniculata butanol fraction has antifertility effect on male rat reproduction, sexual behavior and epididymal sperm concentration. So butanol fraction of Andrographis paniculata root is a potent spermicide which completely immobilized the one million of rat sperm within 20s.

KEYWORDS: Andrographis paniculata, Spermicidal, Sperm motility, Sperm viability.

INTRODUCTION
Present world population is around 6.46 billion and that of India in particular is around 1.1 billion.[1] One of the critical problems of the developing countries like India is its geometrical increase in the human population. This population explosion will have negative impact on our economic policies and would be simultaneously misbalance our socioeconomic infrastructure. Thus the control of human fertility in the sense of its limitation is the most important and urgent requirement. In this search several potential approaches for induction of infertility have been investigated over a long period, including chemical, hormonal and immunological approaches. However, no suitable method has emerged that is effective and free from side effect.[2,3] Hence, there is a need for development of new fertility regulating drug from medicinal plants because from times immemorial humans have relied on plant products as sources of drugs and therapeutic agents. Over the past twenty years, interest in medicinal plants has grown enormously from the use of herbal products as natural cosmetics and for self-medication by the general public to the scientific investigations of plants for their biological effects in human beings. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal products to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people. India has been identified as a major resourceful area in the traditional and alternative medicines globally. In recent times due to low toxicity and long standing experience of exposure, these drugs are used in ethnic medicine system like Ayurveda. Andrographis paniculata (Burm.f.) Wall. ex Nees., (Family- Acanthaceae) (English name-King of Bitters, Tamil name-Nilavempu) In Traditional Chinese Medicine, Andrographis paniculata is a bitter and ‘cold property’ herb. It is used in the treatment of ‘hot’ conditions such as acute infections and fever, including throat infection, pneumonia, tonsillitis, dysentery, gastroenteritis and pylonephritis.[4,5] It is also prescribed for snakebite.[6,7] It is used in Malaysian folk medicine for diabetes and hypertension[2] reported that A.
Panax paniculata had pharmacological properties which include antibacterial, immunological, antiviral, and antithrombotic properties. More recently, A. paniculata has been used in the treatment of chronic bronchitis, administered via aquapuncture, i.e., the injection of an infusion into acupuncture points.[8] In Ayurvedic medicine, it is used as a bitter tonic and stomachic, for diabetes, debility, hepatitis, and as an anthelmintic.[9] A. paniculata extract has been used in different forms, such as tablet or injection. In China, in tablets form it has different names: “Kan Jang” tablets, “Chuanxinlian” tablets, “Xiaoyan Lidan” tablets and “Chuanxinlian antiphlogistic” Pills.[10] The injection forms are “Yamdepieng” and “Chuanxinlian Ruangas”.[11] In Indian pharmacopoeia, 26 Ayurvedic formulations are widely used. In the Unani system of medicine it is considered aperients, antiinflammatory, emollient, astringent, diuretic, emmenagogue, gastric and liver tonic, carminative, antihelminthic and antipyretic. Due to its blood purifying activity it is recommended for use in cases of leprosy, gonorrhea, scabies, boils, skin eruptions and chronic and seasonal fevers.[10,13,14] The characteristic secondary metabolites encountered in this plant have considerably enhanced its importance in the arena of medicinal plants. In this search Andrographis paniculata have antifertility properties which are described in Ayurveda. It has beneficial effect in treatment of wide range of disorder as digestive ailments. In this sequence A. Paniculata root extract of butanol fraction was daily orally fed for two month to study its effect on reproductive function of male albino rats. It was observed that control albino rat showed 100% fertility rate. A. Paniculata root extract butanol fraction, the antifertility effect was 70%. This study was carried out to evaluate in vitro spermicidal & in vivo antifertility activity of this extract against male albino rats. Thus the results suggest a possible antifertility property of the root aqueous extract of A. Paniculata in male albino rats.

MATERIALS AND METHODS

Animal model

Experiment was carried out by using sexually mature albino rats of proven fertility. Animal colonies were developed by breeding animals under normal husbandry conditions. The study will be carry out under the CPCSEA (ICMR, 2006) guidelines will be followed for maintenance and use of the experimental animals.[10]

Preparation of Plant Ethanol extract

A. Paniculata root were collected from local habitat mahabubad, telangana, india. and authenticated Department of Botany, kakatiya university Warangal, and authentication no.is 01487. After identification and authentication, roors of A. Paniculata Linn. Were subjected to drying under shade and then subjected for size reduction to coarse powder by pulverization. The powdered drug was stored in a tightly packed polythene bag.[15]

Extraction

Powdered seeds were charged into cold extraction was carried out using water as solvent. The powdered drug was extracted with solvent until complete extraction. The filtrate was concentrated under the reduce pressure at 50 ± 5°C too obtained extract butanol fraction (15gm) of this plant for experiment.

Treatment protocol for spermicidal activity study

Sperm preparation

After cervical ostiolysis rat epididymis was puncher then, collect semen in incubated normal saline water for in vitro study of rat sperm. Sample has motility (≥50%) and sperm concentration (≥20 million/ml).

Spermicidal activity study

The spermicidal activity was determined by using a modified version of the original protocol (Sander and Cramer method) which measures the minimum concentration of spermicidal agent required to kill 100% sperm within 20s. Test ingredients of various concentrations (2 mg, 4 mg 6mg , 10mg) were mixed with sperm suspension containing 1 million sperm. The mixture was observed under microscope for 20s at 10X and read for motile sperm. The concentration was recorded if any motile sperm were seen. Two – hundred fifty microliter of buffer was added to all the mixture that passed the test and incubated at 37°C for at least 60 minute. The solution was slowly vortexed and observed again for presence of any motile sperm. The concentration at which it was tested was recorded as effective if both test indicated absence of motile sperm. The end point was the lowest concentration of the A.Paniculata root extract butanol fraction that caused complete immobilization of all the sperm within 20 s of mixing. The dose and time dependent study for spermicidal activity was done by using the above test.[16]

Sperm function test

Ability of fertilization is not only dependent on the ovum but also on other sperm functional characteristics. Therefore sperm morphology, motility and viability, reflects the sperm fertilizing capacity was now being increasingly assessed to predict a successful outcome in IVF setting.

Sperm viability test

Sperm were mixed with A.Paniculata root extract butanol fraction separately for 20 s. Sperm viability was checked by using Eosin- Nigrosin technique. Unstained spermatozoa were counted as live sperm and stained spermatozoa were counted as dead sperm.[17]

Treatment protocol for antifertility activity study

Hormonal nature and antifertility effect of the extract butanol fraction were conducted in three experiments. Animals were equally distributed into three treatment groups, each consisting of 6 animals.
**Group A:** Animals of this group were given sterile distilled water alone orally for 60 days. This group was serves as control treated vehicle.

**Group B:** Animals of this group were fed with extract butanol fraction of *A. Paniculata root* at the oral dose of 25 mg/kg body wt/day, for 60 days. Doses were freshly prepared and administrated orally during the study duration.

**Group C:** Animals of this group were fed with extract butanol fraction of *A. Paniculata root* at the oral dose of 50 mg/kg body wt/day, for 60 days. A suspension of the extract butanol fraction of *A. Paniculata root* (50mg/ml) was daily made in distilled water for administration. The required drug was administered orally with a glass syringe fitted with a feeding needle.

**Sperm motility and density**

For determining sperm motility and sperm density, 100 mg of cauda epididymis was minced in 1 ml of physiological saline within a scarification period of 5 minutes. One drop of evenly mixed sample was applied to a glass slide under a cover glass. The sperm motility percentage was determined by counting both motile and immotile spermatozoa per unit area. Next, cauda epididymis sperm density was determined by routine procedure and expressed as million/mm3 of suspension.

**Fertility test**

Successful mating (male:female ratio 1:2) was carried out with all the animals, five days prior to sacrifice period. The mated females were allowed to complete the gestation period. The numbers of pups delivered, litter size and fertility percentage were recorded.

**Body and organ weights**

The initial and final body weights of the animal were recorded. Then the testes, epididymis, seminal vesicle and ventral prostate were dissected out, freed from adherent tissue and weighed accurately up to milligram level.

**Serum biochemistry**

Serum was isolated and stored for detection of protein content, total cholesterol, phospholipids, alkaline phosphatise and LDH by respective calculations.

**Hormone assay**

Blood samples were collected for estimation of serum testosterone, FSH and LH by using radioimmunoassay. Serum samples were separated by standard procedures and stored at 20°C for subsequent analysis. Serum levels of testosterone, FSH and LH were assayed in duplicate by using radioimmunoassay kit.

**Hematology**

The blood samples were collected from the heart and analyzed for blood urea, blood sugar, RBC, WBC and hematocrit levels.

**Statistical Analysis**

Data are expressed as mean ± S.E. and analyze for statistical significance by using student's "t" test. The data are considered as significant at p ≤ 0.01 and non significant at p ≤ 0.001.

**RESULTS**

**Spermicidal activity test**

The spermicidal activity of graded doses of the extract was studied in vitro by using rat semen. The results of Sander–Cramer test showed potent activity of *A. Paniculata root butanol fraction*. The minimum effective concentration of extract butanol fraction required to kill 1 million sperm in 20s was around 10 ± 0.066 (Table 1). For the positive control spermicidal activity test, used the normal saline, there was no motility changes observed (data not shown). The results revealed that with an increase in concentration, there is linear decrease in motility percentage. Approximately 10 mg of extract was required for 100% immobilization of one million sperm in 20 s.

**Sperm morphology**

The morphological; study of sperms were done by using Eosin–Nigrosin stain and no morphological changes were found in sperms head mid-piece or tail when compared with untreated sperms.

**Antifertility studies Sperm motility and density**

Caudaepididymal sperm motility was significantly diminished in the dose regimens. The dose regimens also produced a significant reduction in caudaepididymal sperm density (Table 2).

**Table 1:** Minimum effective concentration (MEC) of extract butanol fraction extract of *A. Paniculata root* stem required to kill 1 million sperm in 20 s.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>semen sample sperm count (million/ml)</th>
<th>Amount of semen taken containing 1 million sperm (µL)</th>
<th>% Motility</th>
<th>MEC % (mg) treatment</th>
<th>Motility after MEC (mg) treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>26</td>
<td>76</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>24</td>
<td>85</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>27</td>
<td>75</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>28</td>
<td>64</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>36</td>
<td>55</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>26</td>
<td>59</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

MEC (mg) mean ± SD 12.00 ± 0.26.

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Table 2: Caudaepididymal sperm motility and density levels after 60 days treatment with extract butanol fraction of A. Paniculata root in male rats.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sperm motility%</th>
<th>Sperm density(million/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A Control</td>
<td>70 ± 0.85</td>
<td>60.6 ± 0.15</td>
</tr>
<tr>
<td>Group B 25 mg /kg b.wt./day</td>
<td>61 ± 0.18b</td>
<td>45.1 ± 0.24 a</td>
</tr>
<tr>
<td>Group C 50 mg /kg b.wt./day</td>
<td>39.01 ± 0.18c</td>
<td>30.07 ± 0.48c</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of 6 animals, Groups B and C was compared with Group A, a Significant (p≤0.05), b Non-significant, c highly significant.

Table 3: Fertility of male rats in control and 25, 50 mg /kg body weight treated with extract butanol fraction of A. Paniculata root for 60 days when mated with females (male :female ratio 1:2).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of females delivering</th>
<th>No of pups</th>
<th>Percent fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A Control</td>
<td>15</td>
<td>52</td>
<td>112</td>
</tr>
<tr>
<td>Group B 25 mg /kg b.wt./day</td>
<td>10</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>Group C 50 mg /kg b.wt./day</td>
<td>05</td>
<td>15</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 4: Body and organ weight after 60 days treatment with butanol fraction of A. Paniculata root in male rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (Control)</th>
<th>Group B (25 mg /kg b.wt./day)</th>
<th>Group C (50 mg /kg b.wt./day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (gm.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>166 ±0.54</td>
<td>164.10±0.54</td>
<td>165.13 ±0.53</td>
</tr>
<tr>
<td>Final</td>
<td>206.06±0.29</td>
<td>205.00±0.18</td>
<td>201.07±0.07</td>
</tr>
<tr>
<td>Organ weight (mg / 100 gm.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testes</td>
<td>704±10.80</td>
<td>504.12±11.13c</td>
<td>401.11 ±0.44c</td>
</tr>
<tr>
<td>Epididymis</td>
<td>315±10.17</td>
<td>156.10±10.18c</td>
<td>133.60±1.02c</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>322±10.17</td>
<td>218.71±1.12c</td>
<td>137.03 ±11.14c</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of 6 animals, Groups B and C was compared with Group A, a Significant (p≤0.05), b Non-significant, c highly significant.

Fertility
A dose dependent reduction in the fertility was observed in treated group. The fertility in the 50 mg/kg body wt/day of A. Paniculata root butanol fraction was70% control by following 60 day of treatment. There was a mark declined inups delivered in treatment group as compare to control group. All the delivered pups were normal and healthy (Table 3).

Body and organ weight
The weight of testes, epididymis and seminal vesicle were decreased significantly. However the weight of the ventral prostrate, Heart, Liver, Kidney and Adrenal gland was non-significantly decreased in all treated animal (data was not shown) while dose regimen did not altered body weight of the animals when compared with control group animals (Table 4).

Serum biochemistry
Cholesterol, protein phospholipids, alkaline phosphates and LDH levels in serum of all treated group were non significantly low after treatment of 25 and 50 mg /kg body weight of A. Paniculata root butanol fraction.

Hormone levels
Testosterone and Follicular stimulating hormone levels was not significantly decreases, while level of Luteinizing hormone was non-significantly in the serum of treated group animals (Table 5).

Hematology
No appreciable alterations were observed in hematological parameters in animals of treated group in comparisons to control group.

DISCUSSION
The result of this investigation demonstrated that the butanol fraction interferes with the structure and function...
of major elements of male fertility as reflected by a marked decrease in the rate of fertility. On the basis of LD50 of A.Paniculata root, selected dose in present study provide the information on the mechanism of toxic action and provide data on which user risk-benefit relationship may be assessed. The plant based contraceptive, inhibit male fertility so after administration of (25, 50 mg /kg body weight) A.Paniculata root butanol fraction exhibited a marked reduction in counts and motility of caudaepididymal sperms in dose dependent manner. The sperm density was decreased concurrently with an increase in the percentage of nonmotile and mature sperms. This could be caused by androgen deprivation. There was decrease in both testicular and epididymal sperm count after chronic administration of drug and this suggested that inhibit may affect androgen binding secretion by sertoli cell via its action on FSH. The reduction of sperm density is also confirmed by histological and hormonal investigation of testis and serum along with fertility status of the animals. The testes of the treated animals revealed the arrest of spermatogenesis. The testicular weight loss is due to the absence of spermatogenic stages particularly in spermatid and spermatozoa, which is further due to decrease in level of testosterone. The testicular damage occurred due to decrease in the seminiferous tubules diameter and in the volume of Leydig cell. Their atrophy is due to inhibition of hypothalamus hypophyseal axis. Since the Androgen binding protein is required for maintaining intra-tubular androgen concentration for cytological differentiation. The loss of sperm motility and structural defects exerted by steroids administration is well known by changing their membrane permeability. These observations indicate that there is a strong interaction between the ethanol extract and plasma membrane of sperm cell. The low cauda EP, low testicular sperms counts, presence of non-motile spermatozoa and significant reduction in the organ weight imply that extract induced infertility might be consequence of an array of factors. One of these factors may be that, these extract interfere with enzymatic reaction including an oxidative phosphorylation uncoupling. This oxidative phosphorylation is required for the ATP which in turns is responsible for sperm motility; slight reduction in ATPase leads to motility inhibition and thus causes infertility. After treatment of A.Paniculata root butanol fraction was no significant changes observed in serum biochemical tests. However in the fertility test, there was reduction in fertility (number of pups) which indicating the fertilization might be due to stored epididymal sperms.

CONCLUSION
Oral administration A.Paniculata root butanol fraction at 50 mg /kg body wt/day in male albino rats did produce antifertility effects. Further long term studies are warranted for evaluation of complete reversible sterility mechanism of action and detailed structural elucidation and toxicological screening with butanol fraction.

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