ABSTRACT
Benign prostatic hyperplasia (BPH) involves the proliferation of epithelium and fibrous muscular tissue of the prostate, commonly seen in aged men. Complications with surgical prostatectomy and side effects with conventional therapy exclude their use as routine treatments for BPH. So, phytotherapy has gained momentum as an alternative. With this background, a standardised polyherbal formulation was subjected for evaluation for its efficacy against BPH. The pharmacological investigation both in vitro (evaluation using NIH 3T3 cell lines by MTT assay) and in vivo (activity against testosterone induced experimental hyperplasia in male wistar rats) were performed to evaluate the efficacy of the polyherbal formulation. From the in vitro study, IC50 of the herbal formulation was found to be 211.8μg/ml and the study showed the better inhibition of cellular proliferation of stromal cells. The study of antibacterial activity showed a good zone of inhibition against E.coli. Data obtained from the in vivo study suggests that the formulation has good efficacy against Benign prostatic hyperplasia in male wistar rats on concentration dependent manner. The polyherbal formulation may be evaluated in clinical trials against benign prostatic hyperplasia.

INTRODUCTION
Benign prostatic hyperplasia (BPH) is a common cause of significant lower urinary tract symptoms (LUTS) in men and is the most common cause of bladder outflow obstruction in men aged above 70 years. BPH affects, to variable degrees, both glandular epithelium and connective tissue stroma of the prostate. The etiology is not clearly understood and is probably multifactorial, but it is well recognized that the condition is related to the aging process and androgenic influence. The complications of BPH include frequency of micturition, nocturia, dysuria and urgency or urge incontinence. Acute retention of urine is an unfortunate obstructive complication. Surgery is the ultimate solution, but pharmacotherapy is often tried first and continued for as long as feasible[1]. The allopathic medicines used in the treatment of BPH are associated with a number of side effects (eg. Finasteride shows sexual complaints like erectile dysfunction) and post-surgical complications are more with the surgical procedures like Prostatectomy. So, phytotherapy has gained momentum as an alternative for the management of BPH worldwide. Medicinal plants like Serenoa repens, Pygeum africanum, Echinacea purpurea, Curcubita pepo, Secale cereal, Hiponis rooperi, Urtica diocia etc. are used widely as an alternative to conventional therapy[2]. A standardised polyherbal formulation in a convenient dosage form along with a scientific validation would contribute significantly in the treatment of BPH[2]. The present study evaluates the pharmacological efficacy of the polyherbal formulation against benign prostatic hyperplasia using in vitro and in vivo studies.

MATERIALS AND METHODS
Composition of the polyherbal formulation
The constituent plants of the formulation were procured and were identified by R & D centre of TTK Healthcare Limited, Chennai. A specimen of each plant part was deposited in the herbarium of the R&D centre. The formulation was developed and standardised. The developed polyherbal formulation was evaluated for its pharmacological efficacy against benign prostatic hyperplasia.

Each capsule contains

<table>
<thead>
<tr>
<th>Herbal drug</th>
<th>Strength</th>
</tr>
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<tbody>
<tr>
<td>Crataeva nurvala</td>
<td>100mg</td>
</tr>
<tr>
<td>Serenoa repens</td>
<td>120mg</td>
</tr>
<tr>
<td>Asperagus racemosus</td>
<td>90mg</td>
</tr>
<tr>
<td>Areca catechu</td>
<td>100mg</td>
</tr>
<tr>
<td>Orchis mascula</td>
<td>25mg</td>
</tr>
</tbody>
</table>

In Vitro Cytotoxicity study using the mouse embryonic fibroblasts cell line (NIH3T3) by MTT Assay [4,5]
Preparation of the polyherbal extractum
Polyherbal capsule granules were successfully extracted with hydro-alcoholic mixture (3:2) for 10 hours using Soxhlet method. Then, the hydroalkoholic extract was centrifuged twice at 1500 rpm for 5 min. Then, the extract was subjected to evaporation on water bath to dryness. The resulted dried extract was used for the in vitro cytotoxicity study.

In vitro cytotoxicity study
The mouse embryonic fibroblasts cell line (NIH3T3) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Dulbecco’s modified eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

Cell treatment procedure
The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of 1x10^5 cells/ml. 100μl of cell suspension was seeded into each of the 96-well plates at plating density of 10,000 cells / well and incubated to allow for cell attachment at 37°C, 5% CO2, 95% air and 100% relative humidity. After 24 hours the cells were treated with serial concentrations of the extracts. The extracts were initially dissolved in dimethyl sulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. 100μl of each concentration was added to the plates to obtain final concentrations of 500, 250, 125, 62.5 and 31.25 μg / ml. The final volume in each well was 200 μl and the plates were incubated at 37°C, 5% CO2, 95% air and 100% relative humidity for 48h. The medium containing no sample served as control. Triplicate was maintained for all concentrations. After 48 hours of incubation, 15μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100μl of DMSO and then the absorbance was measured at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula:

\[
\% \text{ cell Inhibition} = 100 - \left( \frac{A_0}{A_{sample}} \right) \times 100
\]
Nonlinear regression graph was plotted between % cell inhibition and log_{10} concentration and IC_{50} was determined using Graph Pad Prism software.

**In vivo study on the activity against experimental hyperplasia in male wistar rats**[6,7,8]

The protocol for conducting the in vivo study in male wistar rats was approved by the Institutional ethical committee (IEC) of the Madras Medical College, Chennai-600003, India (IEC/3/243 (CPSEA) dated 21.01.2012).

**Experimental animals**

Thirty male wistar rats weighing 180-225 g were selected and randomized into five groups of 6 animals each. The animals were housed in standard laboratory conditions at a temperature of (22±3) °C, relative humidity 50-55 % and 12-hour light-dark cycle. Drinking water and pellet diet (Lipton India Ltd., Mumbai) were supplied ad libitum throughout the study period.

**Experimental design**

The animals received the following treatments:

- **Group I:** Olive oil 1 ml/kg/day, s.c. for 21 days as vehicle control.
- **Group II:** Testoviron Depot (TD) injection 3 mg/kg/day, s.c. in olive oil for 21 days.
- **Group III:** Finasteride 1 mg/kg/day, p.o. and TD 3 mg/kg/day, s.c. in olive oil, both for 21 days.
- **Group IV:** Polyherbal formulation 200 mg/kg/day, p.o. and TD 3 mg/kg/day, s.c. in olive oil, both for 21 days.
- **Group V:** Polyherbal formulation 400 mg/kg/day, p.o. and TD 3 mg/kg/day, s.c. in olive oil, both for 21 days.

[Note: Finasteride and polyherbal capsules were administered orally in the form of freshly prepared homogenized suspension in 0.5%w/v carboxy methyl cellulose (CMC)].

On the 22nd day, the rats were euthanised under diethyl ether anaesthesia. The prostate glands were isolated and the weight of ventral, dorsal and total prostates was recorded. Then, the prostates were fixed in 10% neutral buffered formalin (NBF). The formalin fixed tissues were then processed by paraffin technique and sections of 5 µm thickness were taken and stained by the routine Hematoxylin & Eosin method. The histopathological examination was performed on all the prostate tissues.

**Statistical analysis**

All the results were expressed as Mean ± SD. The results were analyzed statistically using the one way ANOVA followed by Dunnet's test. The minimum level of significance was set at p<0.05.

**Antibacterial study against E.coli** [9]

**Preparation of the extractum**

Granules were extracted with water-alcohol (3:2) for 10 hours using Soxhlet method. Then the hydroalcoholic extract was centrifuged twice at 1500 rpm for 5 minutes. The extract was evaporated on water bath to dryness. The resultant dried extract was used for the antibacterial activity against E.coli.

**Bacterial strains**

Bacterial strains of *Escherichia coli* (ATCC. 8739) used for testing were obtained from HiMedia chemicals. The stock culture was maintained on Mueller Hinton agar medium (HiMedia chemicals) at 37°C.

**Assay for antibacterial testing**

Antibacterial activity of the above mentioned extract was assayed using well diffusion method. Sterilised petri plates containing 10 ml of Mueller Hinton agar medium were seeded with 24 h old culture of *E.coli* strain. A total of 8 mm diameter wells were punched into the agar and filled with the extract. The plates were then incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the zone of inhibition diameter observed. All the tests were carried out in triplicate and their mean value was calculated. The study was done using three different concentrations of the hydro-alcoholic extracts of the polyherbal capsules viz., 50µg/ml, 75µg/ml and 100µg/ml. The results were compared with that of the standard (Ciprofloxacin 10 µg/ml).

**RESULTS AND DISCUSSION**

**In vitro cytotoxicity study of the polyherbal capsules in the mouse embryonic fibroblasts (NIH3T3) cell lines**[10-14]

In the normal human prostate, several cell types are present that are organised in different compartments (epithelial and stromal) and separated by a well-developed basement membrane. The epithelial compartment consists of two main types of cells: basal epithelial cells and glandular secretory cells with numerous neuroendocrine cells, macrophages and lymphocytes dispersed with in this compartment. The surrounding stroma consists of a complex mixture of fibroblasts, myofibroblasts, smooth muscles, endothelial cells and an extracellular matrix. It provides the structural and biochemical support for functional epithelia, thereby defining an optimal microenvironment for epithelial cells. It is well established that under the stimulation of androgens, a close interaction between stromal and epithelial components emerges and this leads to a hyperplastic growth of the tissue.

Due to the non-availability of the normal human prostate (PrEC) cell lines, the mouse embryonic fibroblasts (NIH3T3) cell lines were taken up to evaluate the formulation's efficacy in controlling the stromal cellular proliferation as stroma consists the fibroblasts in more number. The study was carried out using the MTT assay. The herbal extract was tested using the concentrations of 31.25µg, 62.5µg, 125µg, 250µg, 500µg/ml. The results are given in Table 1.

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance</th>
<th>% Cell inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.25</td>
<td>0.14</td>
<td>1.81</td>
</tr>
<tr>
<td>62.5</td>
<td>0.13</td>
<td>10.41</td>
</tr>
<tr>
<td>125</td>
<td>0.11</td>
<td>27.85</td>
</tr>
<tr>
<td>250</td>
<td>0.06</td>
<td>57.24</td>
</tr>
<tr>
<td>500</td>
<td>0.03</td>
<td>82.81</td>
</tr>
</tbody>
</table>

**Fig. 1: Graphical representation of % growth inhibition**

The results show that the hydro-alcoholic extract of the polyherbal capsules shows a dose dependent inhibition of the cellular proliferation in fibroblasts. At the highest concentration of 500µg/ml, maximum inhibition of 82.8% was observed. The IC_{50} value as calculated by the non-linear regression analysis was found to be 211.8 µg/ml.

**Microscopic examination of the cells (Fig.2)**

Control shows a normal architecture of the cells which are polygonal in shape. The cytotoxicity is evident on increasing the concentration of the polyherbal extract. The cells begin to shrink and are rounded with the loss of normal architecture.
In vivo activity against experimental prostatic hyperplasia in male wistar rats

Testoviron depot injection (Testosterone) administration at a dose of 3 mg/kg to rats for 21 days resulted in a significant increase in the weights of the total prostate (about 132.07% increase) as well as dorsal and ventral lobes of the prostate when compared to the normal control (132.07% increase in total prostate). The results are given in Table 2.

Table 2: Effect of polyherbal capsules on prostate weight in testosterone-induced prostatic hyperplasia in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Prostate weight (mg/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total prostate</td>
</tr>
<tr>
<td>I</td>
<td>Vehicle control</td>
<td>109.66±2.43</td>
</tr>
<tr>
<td>II</td>
<td>Testoviron depot (3 mg/kg/day)</td>
<td>254.49±22.89</td>
</tr>
<tr>
<td>III</td>
<td>Testoviron depot (3 mg/kg) + Finasteride (1 mg/kg/day)</td>
<td>167.43±20.89*</td>
</tr>
<tr>
<td>IV</td>
<td>Testoviron depot (3 mg/kg) + Capsules (200 mg/kg/day)</td>
<td>197.14±11.56*</td>
</tr>
<tr>
<td>V</td>
<td>Testoviron depot (3 mg/kg) + Capsules (400 mg/kg/day)</td>
<td>187.01±10.12*</td>
</tr>
</tbody>
</table>

*p<0.05 as compared to Group II. (n=6)
Values in the bracket % reduction of the total prostate weight in formulation treated group when compared to Group II.

Fig. 2: In vitro cytotoxicity study

Fig. 3: Effect of polyherbal capsules on prostate weight in testosterone-induced prostatic hyperplasia in rats. Mean ± SD. (n=6).
In the case of Finasteride treated animals, the weight of the prostate decreased significantly when compared with the Testoviron depot treated group at the dose of 1mg/kg. Finasteride is currently used during the treatment of BPH.

In the case of animals treated with the polyherbal formulation, at both dose levels of 200 mg/kg and 400 mg/kg there was a significant reduction in the weight of prostate gland when compared to the Testoviron depot challenged group. The reduction in prostate weight was dose dependent. The Finasteride treated group shows 34.20% reduction in the total prostate weight when compared to Group II. At the dose levels of 200 mg/kg and 400 mg/kg of the formulation, the reduction was found to be 22.54% and 26.52% respectively. The effect of the higher dose was comparable with the Finasteride treated group. But, in all the treated group animals, the weight of the prostate was higher than the vehicle control group.

The histopathological examinations of the prostates of all groups of animals are shown in Fig. 4.

The histopathological study reports suggest that the groups of animals treated with 400mg/kg of the polyherbal formulation showed reduced hyperplasia and fibrovascular stromal cellular proliferation. The pale eosinophilic materials infiltration into the tissues was also decreased.

**Antibacterial activity against E.coli**

The antibacterial activity of the polyherbal capsules was done against *E.coli* bacteria. Urinary tract infections are the most common in the BPH patients. The main causative microorganism is *E.coli*, which alone accounts about 80-85% of the total infections in the LUTS of the elderly men. Hence, the antibacterial activity of the formulation against *E.coli* was evaluated. The results are given in Table 3 and Fig. 5.

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**Fig. 4: Histopathological examination of the rat prostates**

**Fig. 5: Antibacterial activity against *E.coli***
Table 3: Antimicrobial activity of hydro-alcoholic extract of capsules against E. coli (Zone of inhibition in mm)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Zone of inhibition (Diameter in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formulation</td>
</tr>
<tr>
<td>50</td>
<td>20.63 ± 0.42</td>
</tr>
<tr>
<td>75</td>
<td>22.23 ± 0.25</td>
</tr>
<tr>
<td>100</td>
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</table>

The result indicates that the polyherbal capsules have good antibacterial activity against E. coli. The zone of inhibition as seen with the highest concentration tested i.e., 100µg/ml, was comparable with the standard drug Ciprofloxacin 10µg/ml.

In conclusion, this study demonstrates the efficacy of the polyherbal formulation in reducing the prostatic weight and the stromal proliferation in experimental prostatic hyperplasia in rats. The authors believe that the polyherbal formulation may be passed on to clinical trials in the treatment of benign prostatic hyperplasia and urinary tract infections associated with it after necessary toxicological evaluations.

ACKNOWLEDGEMENT

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REFERENCES