INTRODUCTION

Reproductive health and disorders have always been the crucial issues over generations across all civilization. Of late, the reliance on medicinal plant in solving such problems has been gaining recognition due to its negligible side effect, low cost and easy accessibility. The North-east region of India not only being biodiversity hotspot but also has varied, rich and age old traditional and cultural heritage associated with it. This provides a goldmine of virgin resources that can be explored to gain novel molecules/formulation for therapeutic use. The plant Alpinia galanga also known as Greater galanga, Kalahuan, Kanghoo (English, Hindi and Manipuri respectively) belongs to Zingiberaceae family and has tremendous medicinal property. The rhizome extract of the plant in various solvents has been reported to possess several biological activities, such as antitumor, antimicrobial, anti-inflammatory, antifungal, antiviral, anti-bacterial, gastroprotective, hypoglycemic, hypolipidemic, immunostimulating, anti-leishmanial and apoptosis.

ABSTRACT

Methanolic extract of shade dried rhizome of Alpinia galanga (MAGE) was prepared using Soxhlet apparatus through a minimal of 18 hrs running. Working doses of the MAGE were determined after LD50 study selecting two doses of 200mg/kg Bwt/day and 500mg/kgBwt/day. The ovariectomised mice were randomly allocated into four groups (n=6) viz. vehicle control (1% tween 80), positive control (E2), low dose MAGE (200mg) and high dose MAGE (500mg). After 7 days of oral treatment, animals were sacrificed by cervical dislocation. Sample tissues were carefully collected and weighted using Sartorius balance. Morphological observations revealed that E2 treated group showed positive sign of exogenous estrogenic activity by increasing uterine wet weight and relative increase in uterine/body weight ratio. While low dose MAGE treated group showed weak estrogenic activity by increasing uterine wet weight and uterine/body weight, but less pronounced than E2 treated group. The high dose MAGE treated group showed no estrogenic activity rather showed decrease uterine wet weight as well as morphologically constricted uterine horns which clearly suggests anti-estrogenic activity. The present study provides initial evidence towards establishing either estrogen agonistic or antagonistic hormonal property of MAGE in in vivo animal model.

KEYWORDS: Alpinia galanga, ovariectomy, estrogenicity, anti-estrogenicity.

EVALUATION OF THE EFFECTS OF METHANOLIC EXTRACT OF ALPINIA GALANGA FROM MANIPUR (INDIA) ON UTERUS OF OVARIECTOMISED C3H ALBINO MICE

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INTRODUCTION

Reproductive health and disorders have always been the crucial issues over generations across all civilization. Of late, the reliance on medicinal plant in solving such problems has been gaining recognition due to its negligible side effect, low cost and easy accessibility. The North-east region of India not only being biodiversity hotspot but also has varied, rich and age old traditional and cultural heritage associated with it. This provides a goldmine of virgin resources that can be explored to gain novel molecules/formulation for therapeutic use. The plant Alpinia galanga also known as Greater galanga, Kalahuan, Kanghoo (English, Hindi and Manipuri respectively) belongs to Zingiberaceae family and has tremendous medicinal property. The rhizome extract of the plant in various solvents has been reported to possess several biological activities, such as antitumor, antimicrobial, anti-inflammatory, antifungal, antiviral, anti-bacterial, gastroprotective, hypoglycemic, hypolipidemic, immunostimulating, anti-leishmanial and apoptosis. Earlier phytochemical analysis reveals the presence of alkaloids, saponin, glycosides, terpenoids, phenolics, flavonoids, flavanoids, phytosterols, carbohydrates etc. In addition to having 1’s-1’’-acetoxychavicol acetate as the active pungent principle, Kaempferol, Kaempferide, Galanin and Alpinin are the flavanoids identified from the rhizome. Charles et al., (1992) reported crude oil content of the rhizome as 48% methyl cinnamate, 20-30% cineole, α-pinene, β-pinene and camphor. Traditionally, the Meitei community (non tribal majority community of Manipur, India) has been using the plant Alpinia galanga in various gynecological disorders viz. as abortifacient, during parturition, enhancing male fertility. Our enthnomedicinal survey conducted during 2009-2010 in different part of Manipur revealed that the aqueous extract of freshly grind rhizome of Alpinia galanga with three pinches of salt taken half a glass for 3 days can abort 2-4 month old pregnancy. However despite such traditional claimed there has been no published scientific evidence that can either substantiate or refute such claimed. Therefore, the present study was conducted to provide preliminary scientific evidence in in vivo animal model, taking the estrogen sensitive parameters like uterine wet weight, ratio of uterine weight to body weight and external uterine morphology. The experimental design was logical as estrogen being principle feminizing steroid hormone, maintains and regulates the normal metabolism of female reproductive system. Any alteration, disruption or modulation by plant derived bioactive molecule can be manipulated for potential biological intervention.

MATERIALS AND METHODS

Plants Materials and Extract Preparation

The whole plant Alpinia galanga (Zingiberaceae) were collected from Imphal East, Manipur during July 2009 to April 2010 field visits and authenticated by Prof. P Kumar Singh, taxonomist from Department of Life Science, Manipur University, Imphal, Manipur. Shade dried rhizome powdered (10g) were extracted with methanol in a Soxhlet apparatus for a minimum of 18hr. The methanol from the extract was allowed to get evaporated to yield a concentrated semi-solid mass. The yield of the dried extract from the starting crude material was 20% (w/v). The concentrate was dissolved in 1% tween 80, referred to as MAGE and was used for the present study.

Animals Used

Adult female albino mice (C3H) strain of average body weight (20±2g) and age groups were procured from Animal House Facility of Department of Zoology, Gauhati University, Assam, India. Animals were acclimatized to normal environmental conditions in the laboratory for one week. Standard pallet diet with vitamins and mineral supplements (supplied by Agrivet Farm Care Division, Glazo Smithkline, Chennai, India) and water was given ad libitum. Estrous cycle was observed everyday by microscopic examination of vaginal smear. Only mice showing four consecutive regular cycles were considered for the present study.

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ABSTRACT

Methanolic extract of shade dried rhizome of Alpinia galanga (MAGE) was prepared using Soxhlet apparatus through a minimal of 18 hrs running. Working doses of the MAGE were determined after LD50 study selecting two doses of 200mg/kg Bwt/day and 500mg/kgBwt/day. The ovariectomised mice were randomly allocated into four groups (n=6) viz. vehicle control (1% tween 80), positive control (E2), low dose MAGE (200mg) and high dose MAGE (500mg). After 7 days of oral treatment, animals were sacrificed by cervical dislocation. Sample tissues were carefully collected and weighted using Sartorius balance. Morphological observations revealed that E2 treated group showed positive sign of exogenous estrogenic activity by increasing uterine wet weight and relative increase in uterine/body weight ratio. While low dose MAGE treated group showed weak estrogenic activity by increasing uterine wet weight and uterine/body weight, but less pronounced than E2 treated group. The high dose MAGE treated group showed no estrogenic activity rather showed decrease uterine wet weight as well as morphologically constricted uterine horns which clearly suggests anti-estrogenic activity. The present study provides initial evidence towards establishing either estrogen agonistic or antagonistic hormonal property of MAGE in in vivo animal model.

Keywords: Alpinia galanga, ovariectomy, estrogenicity, anti-estrogenicity.
Toxicity Study
LD50 was determined by the method of Horn, (1956)25 and following Organization for Economic Cooperation and Development (OECD) guideline for testing chemicals section 423(2001).26 The LD50 of MAGE was found to be 4998mg/kg in mice by oral administration. Plant or plant product with LD50 values higher than 2000-3000mg/kg were considered as non toxic.25,27 This supported the logical usage of the plant in traditional medicinal practice.

Chemicals
All chemicals were supplied by North East Chemicals Ltd. Guwahati, India and by Himedia, Guwahati, India through the Department of Zoology, Gauhati University, unless specified.

Experimental Design / Animal Experiment
The mice were weighed and subjected to complete bilateral ovariectomy following the method used by Kalita et al., (1998).28 Briefly animals were exposed to mild anesthesia ketamine (50mg/kgBwt) / xylazine (10mg/kg Bwt) in the ratio 2: 1, through intramuscular injection. A small 1cm incision was made on the dorsal side just above and parallel to the lumbar vertebrae. The ovary from both sides beneath the fat pads was removed one at a time and the incision was stitched immediately. The animals were allowed to recover for at least 3-4 weeks before experimental treatments were started. This creates an animal model for menopause during which endogenous circulating Estradiol (E2) concentration was negligible and the estrogen sensitive target tissues were in their basal state.29 The ovx female mice were randomly allocated into four groups (n=6) viz. control (1% tween 80), positive control (E2), low dose MAGE (200mg) and high dose MAGE (500mg). Tween 80 (1%, v/v, 0.1ml/mouse/day) in vehicle control, Estradiol (0.1mg/kg Bwt in 0.1ml 1% tween 80/mouse/day) in positive Control, MAGE (200mg/kg Bwt in 0.1ml/mouse/day) in low dose MAGE (200mg) and MAGE (500mg/kg Bwt in 0.1ml/mouse/day) in MAGE (500mg) were orally administered using gastric gavages feeding syringe for 7 consecutive days. The animals were weighted 24hrs after the last treatment and sacrificed by cervical dislocation under mild anesthesia (diethyl ether). The uteri, with its luminal fluid intact, were dissected free of adhering fat, mesentery, cervixes and were weighed using Sartorius balance. Then uteri were photographed using Sony Digital Camera (Sony Cyber Shot DSC_W310) for morphological examination. The ratio of uterine to body weight ratio was calculated for each animal using the method of Yamasaki et al., (2000).30 The study was carried out following approval from the Institutional Ethical Committee on the use and care of experimental animals (Gauhati University, Assam, India).

Statistical Analysis
The results were expressed as mean of 6 replicates +/-SEM. Means were analyzed using a one way ANOVA and values of p< .05 were considered statistically significant (Mahajan, 1997).31

RESULTS
The treatment of ovariectomised (ovx) adult mice with E2 or two different dose levels of MAGE for 7 consecutive days showed changes either in absolute uterine wet weight or uterine/body weight ratio in relation to ovx control group respectively (Fig.1; Fig.2). External morphology of the uterine horn in all the experimental groups showed visible changes with respect to the control group (Fig.3). As expected, the E2 treated group showed an increase in both the uterine wet weight as well as uterine/body weight ratio as compared to control group at statistically significant p-value (p<0.0001). The low dose MAGE (200mg) group also showed an increase in the absolute uterine wet weight and uterine/body weight ratio from the control group (p<0.0001) but less pronounced than the E2 treated group. Interestingly, the high dose MAGE (500mg) group showed unexpected results by a decrease in uterine wet weight and uterine/body weight ratio significantly lower than the basal control group. E2 treated group showed visible increase in size from the control group. Both the two dose levels of MAGE showed an elongation of the uterine horns but high dose MAGE (500mg) showed prominent constricted uterine horns as compared to all the other experimental groups.

DISCUSSION
Different plant extracts that are being used for fertility control, as fertility enhancer, or in treatment of post menopausal symptoms and any other gynecological disorders have been shown to possess either estrogenic or anti-estrogenic property.32 Despite common use of the plants in traditional folklore medicine, to our knowledge, there are no reports in literature which evaluate the estrogenic activity of A. galanga rhizome extract. The parameters evaluated in this study are useful indices to assess the potential of a plant as an estrogenic/antiestrogenic agent. One of the early markers of estrogenic action is the fluid imbibition and retention by uterine horn due to enhanced microvascular permeability.33 This along with increases in protein synthesis, mitotic index etc. may contribute to increase uterine weight.34 A classical mouse uterotrophic assay measures an increase in uterine wet weight of ovx mice after exposure to test substance.35 The result of the present study demonstrated that the low dose MAGE (200mg) has potential estrogenic activity as evident by a statistically significant increase of uterine wet weight in the treated dose. The present study reveals that the estrogenic activity of this plant extract was found to be very low as compared to that of E2. Further studies involving graded dose at levels lower than the lowest dose of the present study might be helpful in more accurate determination of estrogenic potential of the MAGE with respect to Estradiol.

Of particular significance is the surprising negative uterotrophic response by high dose MAGE (500mg) treated group that contradicts the effects of the threshold dose response model which is widely viewed as most dominant model in toxicology. Rather this finding is consistent with Calabrese’s concept of hormetic-like biphasic dose response model characterized by low dose stimulation and high dose inhibition.36 The statistically significant decrease in uterine weight much below the basal level of control group may suggest potential anti-estrogenic property at the tested dose level. In addition morphologically constricted or atrophic uterine response strengthens our findings as far as anti-estrogenic activity is concerned. This can be explained in the light of inhibition of uterine fluid imbibitions by anti-estrogenic substances or estrogen antagonist in the classic fluid imbibitions assay.37 Experimental design involving a negative control group (established anti-estrogen like ICI 182,780) in addition to much higher graded dose levels than the present high dose MAGE (500mg) might yield fruitful results in determining the optimal dose ranges eliciting anti-estrogenic response. The result of morphological parameter in the present study also provides an interesting area of research. As such significant elongation of uterine horn in both the tested dose level of MAGE (but not the other treatment groups) points
towards possible role in early stage preparation of implantation reaction. Estrogen primed uterus is a prerequisite for progesterone activity that initiates and maintains decidual cell reaction at the implantation sites.\textsuperscript{28} Length of uterus in polytocous species like mice increases at the time of implantation, to accommodate multiple blastocysts along the length. Endometrial hyperplasia around areas of multiple implantation sites is responsible for the increases in length.\textsuperscript{39} Therefore an in vivo implantation assay along with assessment of progesterogen property of the MAGE in future might be a rational choice. Overall, the results of the present study for the first time demonstrated the estrogenic potential of the rhizome extract of \textit{A. galanga} at the tested dose of 200mg/kg Bwt. Although ovx mice uterotropic assay has been the gold standard for determination of estrogenicity of a substance\textsuperscript{26,37,40} yet assessment of other estrogenic end points cannot be rule out in establishing the molecular mechanism of action of the MAGE.

As such the present study also for the first time provides initial evidences towards determining anti-estrogenic potential of the \textit{A. galanga} rhizome extract. The MAGE eliciting such versatile response may in part be attributed to the presence of wide ranges of phytochemical and essential oils etc. as mentioned previously. The consistent results of the three parameters in all the experimental groups suggest negligible errors in conducting the present study. The traditional claimed of the use of \textit{A. galanga} by Meitei community (Manipur, India) has been in part substantiated by our findings along with generation of a plethora of directions for further research. In \textit{vitro} bioassays for determination steroid hormonal property, photochemical analysis using HPLC, molecular approach and in different model, age group, sexes etc. might be helpful not only in elucidating mechanism of action but also in exploring possible bioactive molecules for therapeutic application.

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REFERENCES


**Fig.1.** Variation of uterine wet weight against various experimental groups (n=6). P-value < 0.0001 at 0.05 level of significant.

**Fig.2.** Variation of ratio of uterine wet weight to body weight in different experimental groups (n=6). P-value < 0.0001 at 0.05 level of significance.
Fig. 3. Photo plates showing external uterine morphology of different experimental groups.

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