Effects of *Ocimum sanctum* Gestational Administration on Physiological and Behavioral Aspects of Mice Offspring

Indu Thakur¹, Papiya Bigoniya², P. Uma Devi¹,³

¹Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Idgah Hills, Bhopal, M.P., India.
²DSKM College of Pharmacy, RKDF University, Gandhi Nagar, Bhopal, M.P., India.
³Clinical Hypnotherapy Institute, Trivandrum, Kerala, India.

Authors’ contributions

This work was carried out in collaboration among all authors. Author PUD designed the study, designed the protocol, wrote the protocol, and approved the final version of the manuscript. Authors IT and PB managed the animal study and data analyses of the study. Author IT performed the statistical analysis and wrote the first draft of the manuscript. Author PB managed the literature searches and designed the study protocol. All authors read and approved the final manuscript.

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(1) Dr. Sawadogo Wamtinga Richard, Ministry of Higher Education, Scientific Research and Innovation, Burkina Faso.

(2) B.Puvarajan, Madras Veterinary College, India.

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ABSTRACT

**Aims:** Prenatal maternal stress and anxiety affect the offspring causing low birth weight, decreased motor activity, and developmental delays. *Ocimum sanctum* is cherished as the holiest herb in India and possesses strong adaptogenic, memory enhancer, and anti-stress properties. This study aimed to assess the effect of *O. sanctum* leaf aqueous extract on preventing pregnancy related stress impact on the mice offspring.

**Methodology:** Pregnant female mice were treated with *O. sanctum* leaf aqueous extract throughout the gestation period at 100 mg/kg, p.o, while exposed to resident–intruder social stress paradigm. Physiological, morphological, and behavioral aspects of offspring were observed for 6 weeks.

**Results:** Pups of *O. sanctum* treated mothers showed significantly (*P* < 0.05) improved body weight, body length, and head length. *O. sanctum* treatment has significantly reduced eye opening.
and pinna detachment period ($P < 0.05-0.01$), and duration for gaining surface righting and mid-air righting reflex ($P < 0.05$). O. Sanctum has significantly increased ($P < 0.05-0.01$) number of correct entries in radial arm maze and line crossing in open field performance.

**Conclusion:** The study outcome proves the beneficial role of O. Sanctum in ameliorating prenatal stress and anxiety induced deleterious effect on offspring.

**Keywords:** Anti-stress; motor behavior; Ocimum sanctum; offspring; prenatal stress.

**ABBREVIATION**
- ANOVA: Analysis of variance
- AOAC: Association of Official Agricultural Chemists
- cGMP–PKG: Cyclic guanosine monophosphate dependent protein kinase G
- CPCSEA: Committee for the Purpose of Control & Supervision of Experiments on Animals
- HPA: Hypothalamo-pituitary–adrenal
- O. sanctum: Ocimum sanctum
- SEM: Standard error of mean
- USA: United States of America

**1. INTRODUCTION**

Stress is physiological, psychological, and behavioral responses related to the perception of the external or internal environment and maintaining equilibrium. Inability to maintain balance results in the experience of distress and anxiety, which, over a period of time, leads to ill health [1]. Symptom of stress includes fatigue, irritability, apathy, anorexia, insomnia, emotional instability, anxiety, and depression. Stress induces autonomic, visceral, immunological, and neurobehavioral responses along with activation of the hypothalamic-pituitary-adrenal axis resulting in elevated corticosterone levels [2]. The stress and anxiety that women experience while pregnancy can affect their health and the health of the offspring causing some problems like low birth weight, earlier delivery, and postpartum depression [3]. Data suggested significant behavioral decrement in rat offspring whose mothers were exposed to stress during the pregnancy [4]. Thompson found that maternal fear during pregnancy resulted in a significant decrease in rat offspring activity [5]. Immune stress during late pregnancy reduced gestational length and negatively impacted birth outcomes, hippocampal function, and central neurosteroid formation in the rat offspring.

Ocimum sanctum is popularly known as holy basil (Krishna Tulsi), belonging to the family Labiate. All parts of O. sanctum like roots, stem, leaves, flowers, and seeds possess medicinal properties and diverse healing properties. Tulsi, the Queen of herbs, is considered one of the holiest and most cherished for its religious and spiritual sanctity in India. This plant has an important role in the traditional Ayurvedic and Unani system of holistic health and herbal medicine of the East. O. sanctum is considered to be an adaptogen, balancing different processes in the body, and helpful for adapting to stress [6]. O. sanctum leaves are helpful in sharpening memory, curing fever and common cold act as an anti-stress agent with anti-convulsions potential, normalizing neurotransmitter levels in the brain which Influences the neurochemistry of the brain [7].

The O. sanctum ethanol extract has protected from adrenergic neurotransmitters loss in rat brains exposed to swimming stress and gravitational stress [8]. Pre-treatment with the O. sanctum extract normalized noise induced changes in neutrophil functions, indicating the stress alleviating effect [9]. Kumara et al. reported effects of O. sanctum leaf extract on restraint stress induced behavioral deficits in male rats [10]. Effects of ethanolic extract of O. sanctum leaf was reported for anti-stress, and adaptogenic activity in male mice against swim endurance test and cold restraint stress [11]. The damaging effects of gestational stress on offspring's behavioral, physiological, and neural development have been well documented [12]. It is proposed to explore the effect of prenatal treatment of pregnant mice with O. sanctum leaf extract on the possible reduction of stress related adverse effects on offspring. Accordingly, the aim of the present study is to evaluate the effects of O. sanctum leaf on preventing pregnancy related stress and its impact on the offspring of Swiss albino mice. This study emphasizes on evaluation of the physiological, morphological, and behavioral aspects of offspring delivered by female mice treated with O. sanctum leaf aqueous extract throughout the gestation period at a dose of 100 mg/kg.
2. MATERIALS AND METHODS

2.1 Plant Collection and Extraction

Fresh leaves of *O. sanctum* were collected from the campus of Jawaharlal Nehru Cancer Hospital and Research Centre, Idgah Hills, Bhopal, Madhya Pradesh. Leaves were washed, shade dried, and powdered. The aqueous extract was prepared by refluxing leaf powder with 3:1 (w/v) double distilled water for 1 hr at 80°C. The supernatant was filtered and condensed in a vacuum drier Speed Vac SC 110A Thermosavant USA, and the yield was calculated.

2.2 Physio-Chemical Analysis

Moisture content was determined as per AOAC method (1965).

2.3 Experimental Animals

Swiss albino mice were obtained from the Department of Research, Jawaharlal Nehru Cancer Hospital, and Research Centre Idgah Hills, Bhopal, Madhya Pradesh. All animals were maintained under standard laboratory conditions of artificial 12 hr light/dark cycle (lights on from 08:00 to 08:00 hr), optimum light 300Lux with an ambient temperature of 21 ± 1°C, and relative humidity of 55 ± 5%. Animals were housed in polypropylene cages with nesting material (autoclaved paddy husk). The mice were given a high protein diet (Golden feed New Delhi) during gestation and postnatal periods and after weaning along with acidified water pH 2.5-3 ad libitum.

2.4 Experimentation

At seven weeks of age, specified pathogen free males and females were used for breeding at 11 weeks of age (1 male × 2 females, per cage). Adult female mice were exposed to a male mouse for a single dark cycle. The day after mating, females were checked for vaginal plugs, placed into a separated home cage, and subsequently weighed twice a week to confirm pregnancy. Males were removed from the cage as soon as pregnancy was confirmed. Chosen pregnant females were divided into 3 groups of 2 each. Group 1 (control) and Group 2 (stressed) was treated with 0.3 ml double distilled water, and group 3 (stressed-treated) was administered orally with 100 mg/kg aqueous extract of *O. sanctum* throughout the estrus cycle.

This study utilizes the resident–intruder as stress paradigm devised by Brunton and Russell [13]. In this model, an ‘intruder’ is transferred into the home cage of a ‘resident’ (usually a same-sex unfamiliar nonspecific) for a specified period of time. Pregnant females of groups 2 and 3 were exposed to social stress by placing them into a cage with an unfamiliar lactating mother. The pregnant ‘intruder’ rats were exposed to a lactating ‘resident’ (lactation days 2–8) for 10 min/day for five consecutive days from days 16 to 20 of pregnancy. This model of prenatal social stress is reported to have increased adrenocorticotropic hormone levels and corticosterone secretion. Group 1 mothers were maintained in their home cage without subjection to the intruder stressors throughout the duration of their pregnancies.

Four to eight litters were born on gestation days 20–21 (counting the day of plug detection as gestation day 0 and the day of birth was considered day 0. The offspring of all three groups were raised by their biological mothers till weaning. The dams were individually housed with the single litter until the pups were weaned. The offspring was studied for postnatal mortality, growth, physiological markers, and behavioral effects. The pups were weaned on day 21 and housed in groups of 4–5 according to gender. Littermates were randomly distributed among cages according to their date of birth, with animals in the same cage not differing by more than 2–3 days in age. Behavioral parameter testing was performed at one month's age.

2.5 Study Parameters

The male and female pups from group 1 dams were designated as ‘control’. The male and female pups from group 2 dams were designated as ‘stressed’ and pups from group 3 was designated as ‘stressed-treated’ groups.

2.5.1 Developmental parameters

The offspring were observed for 6 weeks for postnatal litter size, mortality, body weight, body length (tip of the nose to the tip of tail), head length (through the ear to nose), head width (through the ear to ear) and tail length (tail base at a right angle to the body to the tail tip). Pups were weighed weekly and examined for developmental milestones [14].

The total number of pups born and the number of stillborn pups born to each group were counted at birth, and percentage mortality was calculated.
2.5.2 Physiological markers

Days of appearance of physiological markers like Pinna detachment, fur development, eye opening, vaginal opening, and testes descent during the early postnatal period was observed [15].

2.5.3 Acquisition of reflexes

Days of acquisition of surface righting reflex, mid-air righting reflex, and negative geotaxis was noted [16]. Beginning at 2 days of age, newborn mice were examined daily for acquisition of reflexes for a maximum of 30 sec. In addition to the “day of first performance” of each behavioral test, the time required to perform surface righting, mid-air righting, and negative geotaxis was measured. For observing surface righting reflex, pups are placed in a supine position and ability to return to the prone position with all four paws on the ground. Mid-air righting reflex was measured by releasing pups upside-down from a height of approximately 60 cm turn right-side-up to land on all four paws on a bed of shavings. Negative geotaxis is the ability of the pups to turn 180 degrees and begin to crawl up the slope when placed head down on a 45° incline. All behavioral tests were conducted between 0900 and 1600 hrs [17,18].

2.5.4 Motor behaviors

The pups were assessed for complex motor behaviors at the age of one month using the open field apparatus and radial arm maze. The open-field apparatus consisted of a circular field of 76 cm diameter and an opaque wall 55 cm high. The floor was marked off into 17 sectors divided by two circles into outer, inner, and central regions. A 60 W light bulb was hung above the center of the open field. Each mouse was individually placed into an external sector of the open field and observed for 5 min. The total number of sectors entered by the animal (at least three quarters of the body), and the number of rearing, grooming, defecation boluses, and urination frequency were recorded over a test period of five minutes. The floor of the open field was cleaned with water before placing another animal [19,20].

The radial arm maze consisted of a polypropylene central octagonal arena with eight radial arms. The central arena is 32 cm, width 4 cm, height 4 cm, and the central diameter is 22 cm. The entire maze was set on a circular turntable, and lighting was provided by very low emitting Lux near the maze. At the start of each session, 1-2 mg reward pellets were placed in each of the eight food wells. The mice were placed in the central arena, and all the doors were raised. The mice were deemed to have entered an arm when all four paws were in that arm. All doors were lowered, and the mice were allowed to eat the reward pellets in that arm. The door to that arm was then raised briefly, so allowing the mice back into the central arena where it was confined for approximately 10 sec. Mice were removed when all the eight arms had been visited. However, the session was stopped if the mice had not completed the task after 10 min or if the delay between selecting arms was more than 2 min [21].

2.6 Statistics

Data were analyzed using Microcal Origin software (version 0.7) by a one-way ANOVA or t-tests for independent samples. Subsequent comparisons between groups were carried out using Newman-Keuls procedures.

3. RESULTS

3.1 Extraction

The yield of *O. Sanctum* aqueous dried extract was 8-10%. The extract was blackish-brown in color when completely dried. Moisture content was 7.91% and, total ash and water insoluble ash content was 0.86 and 0.42%, respectively.

3.2 Developmental Parameters

Average litter size in both control and *O. sanctum*, treated females were 6-8, whereas in sham stressed, it was 4-6. Pups of control, stressed, and *O. sanctum* treated groups showed 8.50, 14.25, 00% mortality at birth. Stressed pups showed significantly (*P* < 0.05) lower body weight gain, weekly body length, and head length, whereas the effect on the tail length and head width was non-significant compared to control. Six weeks data for postnatal body length, head length, head width, and the tail length of mice pups showed a non-significant difference between control and 100 mg/kg *O. sanctum* aqueous extract treated groups, but body weight gain was significantly higher. Pups of *O. sanctum* treated mothers showed significantly improved weekly gain of body weight, body length, and head length compared to the stressed group (Fig. 1 and Fig. 2).
Fig. 1. Effect of prenatal *O. Sanctum* administration on the postnatal body weight, body length and tail length of mice pups

Values are Mean ± SEM of eight animals per group. *P = .05 compared to vehicle control and aP = .05 compared to stressed group values.
3.3 Physiological Markers

Physiological growth related marker data revealed that eye opening and fur development was significantly ($P = .05$) delayed in the stressed group. Pups from mothers treated with *O. sanctum* showed significantly ($P = .05 \text{ to } .01$) reduced in eye opening and pinna detachment period compared to the stressed group. The effect of *O. sanctum* treatment was non-significant on fur development, vaginal opening, and testes descent durations (Table 1).

3.4 Acquisition of Reflexes

Prenatal stress has caused significantly ($P = .05$) higher duration in pups to gain surface righting reflex and mid-air righting reflex. The time for gaining surface righting reflex and mid-air righting reflex was significantly ($P = .05$) shorter in *O. sanctum* treated female offspring compared to the stressed group. *O. Sanctum* has non-significant effect on time to complete the negative geotaxis task compared to the control group (Fig 3).
Table 1. Effect of prenatal *O. sanctum* administration on the postnatal physiological marker appearance in mice pups

<table>
<thead>
<tr>
<th>Group</th>
<th>Pinna Detachment (M ± SEM)</th>
<th>Fur Development (M ± SEM)</th>
<th>Eye opening (M ± SEM)</th>
<th>Vaginal opening (M ± SEM)</th>
<th>Testes descent (M ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.20 ± 0.17</td>
<td>5.75 ± 0.28</td>
<td>15.25 ± 1.24</td>
<td>21.80 ± 2.54</td>
<td>22.50 ± 2.55</td>
</tr>
<tr>
<td>Stressed</td>
<td>6.15 ± 0.90 *</td>
<td>7.73 ± 1.04 *</td>
<td>16.70 ± 1.68*</td>
<td>22.40 ± 3.25*</td>
<td>23.80 ± 3.66*</td>
</tr>
<tr>
<td><em>O. sanctum</em> (100 mg/kg)</td>
<td>3.43 ±0.22 b, 5.96 ± 0.47**</td>
<td>11.51 ±1.09**</td>
<td>21.25 ± 2.06**</td>
<td>22.75 ± 2.60**</td>
<td></td>
</tr>
</tbody>
</table>

Values are Mean ± SEM of eight animals per group. *P = .05 compared to vehicle control and aP = .05, bP = .01 compared to stressed group values.

3.5 Motor Behavior

3.5.1 Open field performance

Stressed group pups showed significantly (*P = .05*) lower line crossing and higher fecal boli count, grooming, and rearing response. The frequency of crossed grid lines with all four paws was significantly (*P = .05 - .01*) higher in prenatal *O. sanctum* treated offspring than control and the stressed group. Prenatal *O. sanctum* treated offspring showed a normal frequency of fecal droppings and urination. *O. Sanctum* has significantly (*P = .05*) decreased grooming response but not rearing response as compared to prenatal stressed group (Table 2).

3.5.2 Radial arm maze performance

Two types of memories, i.e., working and references, are assessed during the radial arm maze task. The stressed group showed a significant (*P = .05*) reduction in the number of correct entries in all eight arms, whereas the number of correct entries in all arms and the time taken for entries was significantly (*P = .05-.01*) higher in *O. sanctum* treated group (Fig. 4).

4. DISCUSSION

Maternal stress is common adversity during pregnancy correlated to gestational corticosterone alternations, and studies have
Table 2. Effect of prenatal *O. sanctum* administration on the postnatal one month old mice pups performance in open field

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of lines crossed</th>
<th>No. of faeces</th>
<th>No. of Urination</th>
<th>No. of grooming</th>
<th>No. of Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>149.21 ± 10.97</td>
<td>3.60 ± 0.17</td>
<td>0.83 ± 0.009</td>
<td>5.33 ± 0.64</td>
<td>1.85 ± 0.15</td>
</tr>
<tr>
<td>Stressed</td>
<td>113.78 ± 12.45</td>
<td>5.95 ± 0.25</td>
<td>0.97 ± 0.005</td>
<td>7.12 ± 0.33</td>
<td>2.56 ± 0.36</td>
</tr>
<tr>
<td><em>O. sanctum</em> (100 mg/kg)</td>
<td>206.95 ± 7.57</td>
<td>3.55 ± 0.22</td>
<td>0.56 ± 0.24</td>
<td>6.62 ± 0.52</td>
<td>2.72 ± 0.28</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM of eight animals per group. *P = .05 compared to vehicle control and aP = .05, bP = .01 compared to stressed group values.

Fig. 4. Effect of prenatal *O. sanctum* administration on the radial arm maze test of one month old mice pups

Values are Mean ± SEM of eight animals per group. *P < .05 compared to vehicle control and aP = .05, bP = .01 compared to stressed group values.

shown that maternal stress during pregnancy also contributes to the etiology of postpartum behavioral changes. Neuroendocrine and behavioral responses of maternal stress on the offspring are particularly mediated by enhanced hypothalamo-pituitary–adrenal (HPA) axis responses. The days regulation of HPA axis function also interferes with the hypothalamic–pituitary–gonadal axis [22]. Deficits in child development associated with maternal depression are correlated with elevated cortisol levels in the mother, suggesting that the stress hormones play a crucial role in the detrimental impact of maternal depression on overall child development [23].

The main objective of this study was to examine the postnatal growth and behavior of pups, including overall activity level, learning, memory, and motor ability, while the prenatally stressed mothers were treated with *O. sanctum* during the gestation period. The present study implemented an ethologically relevant social stressor model representing the type of stress that pregnant women may practical experience. The effects of social defeat by a resident lactating mouse on the pregnant female were assessed during the last week of pregnancy over 5 days. This protocol is reported to enhance HPA axis response of the pregnant intruder rat, which eventually affects the HPA responsivity towards postnatal stress and anxiety-related behavior of the offspring. Brunton and Russell correlated the increased level of adrenocorticotropic hormone and corticosterone secretion with prenatal stress [13].
The data obtained in the present study indicated that prenatal social stress has significantly delayed physiological development, acquisition of reflexes and motor responses with increased stress and anxiety like behavior. Prenatal stress showed reduced body weight gain in pups whereas gestational *O. sanctum* treatment positively affected the postnatal body weight and growth parameters of mice pups. Preweaning body development of *O. sanctum* treated pups was similar to that of the control group. Stress exposure during pregnancy has detrimental effects on the birth weights of the newborn, as reported [22]. Prenatal stress caused a considerable reduction in pinna detachment, eye opening, and fur development, whereas pups mother treated with *O. sanctum* showed normal physiological markers. *O. sanctum* treated mother litters also showed normal morphological parameters, which were found to be delayed by prenatal stress.

Prenatal stress results in a general syndrome of behavioral change [24]. Offspring of prenatal stress mother showed signs of anxiety, stress, reduced motor ability, and memory deficit. *O. sanctum* treated mice litters improve their performance in successive trials over the 3 days of acquisition testing, and the treated group also significantly differ from the stressed group in the number of correct entries in the radial arm maze, suggesting that the pups were capable of reward conditioning.

Hyperresponsiveness of the HPA axis is associated with postpartum depression, similar to what has been observed in women with postpartum depression. This mediates the adverse effects of maternal depression on offspring behavior. Neuroendocrine, molecular, and physiological adaptations take place during pregnancy, parturition, and the post-partum period. These act in concert to reshape the female brain and modify the behavior showing a high level of maternal responsiveness, mood, cognition, and stress regulation [25]. The HPA axis response to a variety of stressors is severely attenuated towards the end of pregnancy with a concomitant increase in basal corticosterone levels. This adaptive response seems to be essential for the healthy development of the offspring [26]. An unsupported rearing and grooming usually is a more direct measure of anxiety. Offspring of *O. sanctum* mice showed stress reduction normal rearing response, which is considered as a sign of emotionality. A significant increase in both the anxiety and emotionality responses indicates achievement of regular or early maturation compared to control.

Prenatal stress causes postnatal growth retardation and impairment in spatial learning and memory by affecting the hippocampal oxidative phosphorylation and inhibiting the cGMP–PKG pathway in the hippocampus of offspring stressed rats [27]. Prenatal stress decreases dendritic length, spine density, neuron number, hippocampal synapses number, changes in gene expression associated with neural development, cell differentiation, and neurotransmitter function in the brains [28]. Gavin et al. found that up to 13% of women experience depressive episodes at some point during pregnancy [29]. Antenatal psychological distress has a profound impact on fetal behavior and child development [30]. Maternal antenatal anxiety and/or depression are related to increased risk for neuro developmental disorders in children and are associated with poor emotional adjustment in young children [31]. Children of mothers having postpartum depression exhibit deficits in cognitive development, motor, and emotional development [4]. Physical or psychological stress in women during pregnancy can increase the propensity for mild cognitive impairments and behavioral problems in children and increased susceptibility to developing cardiovascular, metabolic, and neuropsychiatric disorders in later life [17, 32, 33].

Maternal stressors in rodents can replicate some of the abnormalities in offspring behavior observed in humans [34]. Prenatal stress during the critical period of fetal brain development correlates with the development of human psychiatric disorders, such as schizophrenia. The second trimester of pregnancy in humans seems to be the most vulnerable period for insult [35]. In the present study, the pregnant mice were exposed to social stress during the third week of pregnancy which is similar to the second trimester of human gestation [36]. About one third of postpartum depression is resistant to chronic treatment of a conventional monoamine-based antidepressant such as fluoxetine, a selective serotonin reuptake inhibitor [37]. Use of antidepressant drugs are considered unsafe and possess a potential risk to the developing fetus, making prenatal stress challenging as they can cross the placenta, increasing the risks of teratogenicity [38]. A sage option can be controlling prenatal stress with herbal remedies. 

Very few herbs like *Valeriana fauriei, Panax*
ginseng and Hypericum perforatum have been scientifically validated for the beneficial role in prenatal stress modalities [39-41]. This study validates potential beneficial effects of O. sanctum consumption on the postnatal development and behavioral pattern of prenatal stressed mice pups.

The use of synthetic as well as herbal drugs during gestation should be monitored carefully for safety profile. Tulsi has been used in Asia for thousands of years and has more recently become one of the most popular herbal supplements in food products, perfumery, dental and oral products. O. sanctum leaf ethanolic extract administered to mice (up to 800 mg/kg orally) for 28 days has not produced any harmful effect on body weight, hematological and biochemical profiles [42]. Gupta et al. reported Ocimum side A and B as major anti-stress phytoconstituent present in O. sanctum [43]. These novel coumarins of O. sanctum are effective against stress and anxiety disorders by modulating the HPA axis and the monoaminergic system along with antioxidant properties [44].

5. CONCLUSION

The present study provides valuable data regarding tulsi’s beneficial role in ameliorating prenatal stress induced deleterious effect on offspring. This study indicates that consumption of tulsi (O. sanctum) daily in optimum quantity during gestation can potentially assist in better physiological growth and development of newborn child. The characterization of the exact mechanism involved in the anti-stress effects as well as the participation of neurotransmitters and receptors could be the subject of further studies.

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DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

Not applicable.

ETHICAL APPROVAL

Health monitoring was performed according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines confirming the specified pathogen free health status of animals maintained in the same animal room. The experimental protocol was approved by the Institutional Animal Ethics Committee (500/01/a/CPCSEA;31/10/2001).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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