

Anti-inflammatory, Anti-estrogenic, and Anti-implantation Activity of *Bergia suffruticosa* (Delile) Fenzl

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ABSTRACT

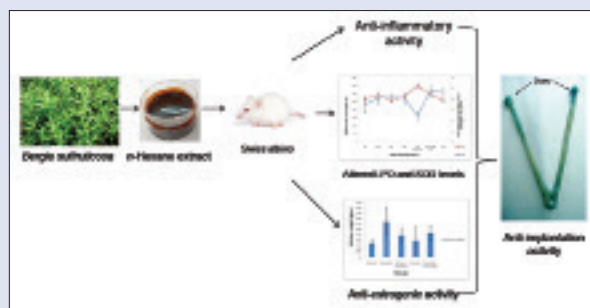
Background: *Bergia suffruticosa* (Delile) Fenzl (Syn. *Bergia odorata* Edgew.) (Elatinaceae family) is used traditionally to repair bones and is applied as a poultice on sores. It is also used for stomach troubles and as an antidote to scorpion stings. So far, very little scientific work has been reported to validate its ethnomedical uses in the alleviation of pain, bone repair, etc., **Objective:** This study was designed to explore the anti-inflammatory and anti-implantation potential of *n*-hexane extract of *B. suffruticosa* whole plant in mice along with identification of its chemical constituents. **Materials and Methods:** *n*-Hexane extract of *B. suffruticosa* whole plant was screened for acute and chronic anti-inflammatory activity followed by an anti-estrogenic activity. Eventually, *n*-hexane extract was tested for anti-implantation activity by exploiting markers of uterine receptivity, lipid peroxidation, and superoxide enzyme activity. The extract was administered orally at a dose of 100 mg/kg body weight in each study. **Results:** Thin layer chromatography fingerprint profile of *n*-hexane extract revealed the presence of lupeol and β -sitosterol. The *n*-hexane extract reduced the edema by 80% in acute inflammation, whereas it reduced edema to 75% on the 5th day in chronic inflammation. The *n*-hexane extract reduced elevated malonaldehyde level from 6 to 2.5 nmol/g $\times 10^{-5}$ and increased superoxide dismutase enzyme activity from 0 to 350 units/g in treated animals on the 5th day of pregnancy. Moreover, extract decreased uterine weight from 0.33 to 0.2 g in estradiol treated animals. **Conclusion:** These results indicate that *n*-hexane extract of *B. suffruticosa* is having potent anti-inflammatory, anti-estrogenic, and anti-implantation activity. This is the first report of all the pharmacological activities of *B. suffruticosa* mentioned above.

Key words: Carrageenan and formalin induced inflammation, lipid peroxidation, lupeol, superoxide dismutase, β -sitosterol

SUMMARY

- TLC fingerprint profile of *n*-hexane extract of *Bergia suffruticosa* whole plant revealed the presence of lupeol and β -sitosterol
- n*-Hexane extract showed *in vivo* anti-inflammatory activity in both acute and chronic model of inflammation in rats
- n*-Hexane extract possess significant anti-estrogenic activity

- n*-Hexane extract altered the levels superoxide anion radical and superoxide dismutase enzyme activity during the blastocyst implantation
- Anti-implantation activity of *n*-hexane extract is attributed to its anti-inflammatory and anti-estrogenic potential.



Abbreviations used: TLC: Thin layer chromatography; LPO: Lipid peroxidation; SOD: Superoxide dismutase; *B. suffruticosa*: *Bergia suffruticosa*; TNF- α : Tumor necrosis factor- α ; NO: Nitric oxide; IL-1: Interleukin-1; LIF: Leukemia inhibitory factor; CSF-1: Colony-stimulating factor; COX: Cyclooxygenase; SDS: Sodium dodecyl sulfate; IAEC: Animal House Ethics Committee; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; HBSS: Hank's balanced salt solution; MDA: Malonaldehyde; and TBA: Thiobarbituric acid.

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INTRODUCTION

Throughout the ages, humans have relied on nature for their basic needs including the treatment of various diseases. Natural products have been the most productive source of lead compounds for the development of novel drugs. This is genuinely true when applied to drug discovery in "olden times" before the advent of high-throughput screening and the postgenomic era: More than 80% of drug substances were of natural product origin or inspired by a natural compound. However, comparisons of the information presented on sources of new drugs from 1981 to 2007 points out that almost half of the drugs approved since 1994 are based on natural products.^[1] Further, due to mild action and fewer side effects natural product based medicines are getting an increased therapeutics market share. According to the World Health Organization report, approximately 80% of the world population prefers plant based drugs for the treatment of various diseases.^[2]

Bergia suffruticosa (Delile) Fenzl (syn. *Bergia odorata* Edgew.) (Elatinaceae) is used traditionally to repair bones and healing of wounds.^[3] Ethnomedical claims also report its use in gastrointestinal

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disorders^[4] and as an antidote to scorpion stings.^[5] The plant is reported to show antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.^[6] The 95% ethanol extract of the whole plant is reported to exhibit molluscicidal activity against *Biomorphalaria pfeifferi* and *Bulinus truncalus*.^[7] Yet, very few reports are published on the pharmacological activity, as well as phytochemical analysis of *B. suffruticosa*. Hence, *B. suffruticosa* is merely an unexplored plant.

Our previous phytochemical study has reported the presence of gallic acid, gallicin, lupeol, and β -sitosterol in the plant.^[8] These compounds have been reported to have anti-inflammatory activity.^[9-12] Another report from our institute stated that the methanol extract of *B. suffruticosa* exhibits good free radical scavenging activity.^[13] Recently, we have also reported the ulcer protective effect of hydroalcoholic extract of *B. suffruticosa*.^[14] All these evidence inspired us to evaluate anti-inflammatory and anti-implantation potential of this plant.

The process of implantation is a very critical event in reproductive physiology and is required for nearly all viviparous births. Many biochemical, biophysical, and hormonal changes occur prior to this event.^[15] One of the crucial aspects in this whole reproduction process is "endometrial receptivity" which is necessary for blastocyst implantation.^[16] The process of implantation is modulated by various "pro-inflammatory" mediators along with the ovarian hormones. Studies have shown that various inflammatory mediators such as tumor necrosis factor- α , nitric oxide (NO), superoxide anion radical, interleukin-1, leukemia inhibitory factor, colony stimulating factor-1, etc., are involved in the process of implantation.^[17-20] Prostaglandins (PGs) are also implicated as an important mediators for increased endometrial vascular permeability during implantation.^[21,22] This phenomenon is evident from delayed implantation or implantation failure by cyclooxygenase (COX)-1 and COX-2 inhibitors in dose dependent manner in mice.^[22] Hence, it is clear that there exists a possibility that any compound showing good anti-inflammatory activity may show significant anti-implantation activity. For instance, *n*-hexane fraction of *Vitex negundo* Linn (Verbenaceae) leaves inhibit blastocyst implantation in mice when administered orally on days 1–6 of pregnancy.^[23] *V. negundo* is very well known for its anti-inflammatory activity. Similarly, leaf extract of *Hibiscus rosa-sinensis* is also reported to possess anti-implantation activity.^[24]

It is also reported that the recruitment of these pro-inflammatory mediators at the site of implantation is modulated by the ovarian steroid hormones (estrogen and progesterone). Pro-inflammatory molecules which are useful during implantation are secreted by leucocytes in response to changes in these hormonal levels,^[17] especially estrogen. Thus, it can be hypothesized that any compound showing good anti-estrogenic activity may also downregulate these pro-inflammatory molecules and inhibit blastocyst implantation.

MATERIALS AND METHODS

Plant material

The whole plant of *B. suffruticosa* required for the experimental work was collected in the month of July 2011, from the campus of the National Institute of Pharmaceutical Education and Research (NIPER), Ahmedabad (housed at B.V Patel Pharmaceutical Education and Research Development [PERD] Centre). The collected plant was identified referring to Flora of Gujarat.^[25] The authenticity of the plant was further confirmed by Dr. Anandjiwala, a plant taxonomist. Herbarium and voucher specimen (NIPER-A/NP/0711/01) of the plant collected have been preserved in the Department of Natural Products, NIPER, Ahmedabad for future reference. The collected plant material

was air dried and then dried in hot air oven at $\leq 40^{\circ}\text{C}$. The dried plant material was stored in an airtight glass bottle at 30°C and powdered to 40 mesh whenever required.

Chemicals

β -sitosterol (purity: 99.19% w/w) was purchased from the Natural Remedies Pvt. Ltd., Bangalore, India and lupeol (purity: 99.51% w/w) was a gift sample from S.C. Pal College of Pharmacy, Nasik, India. Agar was purchased from Merck, Mumbai, India. Pyrogallol (pyrogallol acid; 1,2,3-trihydroxybenzene), sodium dodecyl sulfate (SDS), Chicago blue 6B dye, and 2-thiobarbituric acid (TBA) were purchased from HiMedia Laboratories, Mumbai, India. Triton X-100 and ethinyl estradiol were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Diclofenac sodium was obtained from Troikaa Pharmaceutical Ltd., Ahmedabad, India, whereas dexamethasone was obtained from Cadila Pharmaceuticals Ltd., Ahmedabad, India. Tamoxifen (brand name, Tamtero) was purchased from Hetero Drug Ltd., India. All the solvents used were of analytical grade and obtained from Merck, Mumbai, India. Deionized water used in the experiment was prepared in-house using a water purifier system (Millipore Elix, Germany).

Preparation of *n*-hexane extract of *Bergia suffruticosa* whole plant

Powdered material (250 g) was extracted with *n*-hexane (2 L \times 3) under reflux in a water bath at 70°C for 1 h. The extract was filtered by using Whatman filter paper no. 1, concentrated to dryness using a rota vapor. The solvent was recovered, and the extract was dried under vacuum for further use.

Development of thin layer chromatography fingerprint profile and co-thin layer chromatography of *Bergia suffruticosa* with lupeol and β -sitosterol standard

n-Hexane extract (20 mg) of *B. suffruticosa* was dissolved in 40 ml of *n*-hexane, treated with a pinch of charcoal, filtered and the volume was made up to 50 ml in a volumetric flask. This sample solution was used for the thin layer chromatography (TLC) fingerprinting profile. TLC plates consisted of 10 cm \times 10 cm, precoated with silica gel 60 F₂₅₄ TLC plates (E. Merck) (0.2 mm thickness) with aluminum sheet support. The spotting device was a Camag Linomat V Automatic Sample Spotter (Camag, Muttentz, Switzerland); the syringe, 100 μl (Hamilton). The developing chamber was a Camag glass twin trough chamber (20 cm \times 10 cm); densitometer a Camag TLC Scanner 3 linked to winCATS software (Camag, Muttentz, Switzerland). The experimental conditions were kept constant where, the temperature was $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and relative humidity was 40%. TLC fingerprint was developed by applying 25 μl of *n*-hexane extract (100 mg/50 ml) in a duplicate along with standards, lupeol and β -sitosterol with band size of 8 mm, and distance between the tracks of 12 mm. Plate was developed in a solvent system of toluene: Methanol (9.3:0.7), dried and observed under ultraviolet (UV) 254 nm and UV 366 nm. The plate was derivatized with anisaldehyde-sulfuric acid reagent followed by heating at 100°C until the colored band appeared. The R_f value and color of the resolved bands were noted.

Animals

Sprague Dawley rats (150–250 g) of both sex and female Swiss albino mice (20–30 g) were used for *in vivo* pharmacological testing. All the experimental protocol was approved by the Institutional Animal House Ethics Committee (IAEC), constituted by the Ministry of Social

Justice and Empowerment, Government of India. The animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, vide registration no. 1661/PO/a/12/CPCSEA, dated 21 November 2012. Animal housing and handling were performed according to CPCSEA guidelines. All animals were maintained under standard laboratory conditions at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with relative humidity 45–65% and 14:10 h light and dark cycle. Commercial pellet diet and water were provided *ad libitum*. The animals were fasted overnight prior to each experiment.

Carrageenan induced acute inflammation

Animals were divided into three groups ($n = 6$) normal control, positive control (diclofenac sodium 10 mg/kg), and *n*-hexane extract (100 mg/kg) were kept on fasting for 12–18 h prior to experiment. Animals were dosed with diclofenac sodium (10 mg/kg) or extract (100 mg/kg) 1 h prior to carrageenan injection. All the drugs were orally administered with 0.2% agar suspension freshly prepared as a vehicle. Carrageenan solution (0.1 ml of 1% solution) was injected into the subplantar region of the right hind paw of each rat after 1 h of drug administration. The paw volumes of rats were measured using digital plethysmometer (IITC Life Science, CA, USA) up to 3 h. The inflammation was assessed by calculating the %edema protection by the following equation:

$$\% \text{Edema protection} = [1 - (V_t/V_c)] \times 100$$

V_t = Edema volume in the drug treated group

V_c = Edema volume in the control group.

Formalin induced chronic inflammation

Animals were divided into three groups of 6 animals each and kept on fasting for 12–18 h prior to experiment. Animals were dosed with dexamethasone (5 mg/kg) (positive control) or *n*-hexane extract (100 mg/kg) 1 h before the injection. All the drugs were orally administered with 0.2% agar suspension freshly prepared as a vehicle. The initial paw volumes were measured using plethysmometer and noted as 0 h reading. Formalin solution (0.1 ml of 2% solution, prepared in normal saline) was injected in the subplantar region of the right hind paw of rats 1 h after drug administration. Final reading was recorded 3 h after the injection. This exercise was continued for 5 consecutive days. The degree of inflammation was measured on all 5 days. The inflammation was assessed by calculating the %edema protection according to the following equation.

$$\% \text{Edema protection} = [\text{control} - (Z_2 - Z_1)/\text{control}] \times 100$$

Z_1 = First day 0 h reading

Z_2 = Consecutive day final readings.

Anti-implantation activity

Mature, female mice (Swiss albino strain, *Mus musculus*, 2–3 months old) housed in temperature-controlled ($27^{\circ}\text{C} \pm 1^{\circ}\text{C}$) room at light:dark regimen of 12:12 h were used for the study. The experimental protocol was approved by the IAEC, constituted by the Ministry of Social Justice and Empowerment, Government of India. Only those female mice that showed a regular 4–5 day estrous cycle were used in the study. Vaginal smears were examined daily according to the guidelines provided by Caligioni.^[26] Females that showed a proestrus smear (day 0) were mated with a male of proven fertility on the same evening. The presence of a vaginal plug (next day morning) confirmed mating and was designated as day 1 of pregnancy. Animals were divided into two groups, Groups A and B. Animals from Groups A and B were dosed orally at 10:00 am each day from day 1 to day 6 of pregnancy with 0.2% agar (control), *n*-hexane

extract (100 mg/kg body weight). The pregnant females were sacrificed on days 1–5 (10:00 am), 5 (4:40 am), and 6 (10:00 am) of pregnancy. The uterus was excised from each animal, cleaned from adhering fat and washed with normal saline. Appropriately weighed uterus was used for estimation of lipid peroxidation (LPO) and assay of superoxide dismutase (SOD) activity. A second set of animals ($n = 6$) from each group were injected intravenously 0.1 ml of 1% Chicago blue 6B dye through a tail vein on day 6 of pregnancy. All the animals were sacrificed, and the uterus was exposed to count the number of sites of implantation.

Estimation of lipid peroxidation

Uterine tissue was taken in 5 ml of Hank's balanced salt solution (HBSS, pH 7.4) and homogenized at 5000 rpm (3 cycles of 30 s each), using a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was then centrifuged at 3500 rpm for 10 min. The pellet was resuspended in 0.1 ml of HBSS and used for estimation of LPO. LPO was measured in terms of malonaldehyde (MDA) TBA reaction as reported by Okahwa *et al.*^[27] The reaction mixture contained 0.1 ml of tissue homogenate (as described above), 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with 1 M NaOH), and 1.5 ml of 0.8% aqueous solution of TBA. The reaction mixture was made to a volume of 4 ml with the addition of 0.7 ml of double distilled water and heated at 95°C for 1 h in a water bath. After heating, 1 ml of double distilled water and 5 ml of a mixture of *n*-butanol and pyridine (15:1 v/v) was added and the mixture was shaken vigorously on a vortex mixer for 5 min. This mixture was then centrifuged at 3000 rpm for 7 min. After centrifugation, the upper organic layer was separated and the amount of MDA formed in this layer was measured at 532 nm using a UV/Visible spectrophotometer (Systronics, Ahmedabad, India). Appropriate controls were used at different steps during this estimation (extinction coefficient of MDA is 1.45×10^{-5} /min/cm).

Assay of superoxide dismutase activity

The uterine tissue was taken in 4 ml of chilled Tris buffer (50 mM pH 8.2) and was homogenized at 13,000 rpm (3 cycles of 30 s each), using a Polytron homogenizer. The homogenate was treated with 1 ml of 0.1% Triton X-100 (v/v) for 20 min at 4°C . Homogenate was then centrifuged at 10,000 rpm at 4°C for 30 min using a Sorvall, legend Micro21R centrifuge. The supernatant was used for the assay of SOD activity by the method as reported by Marklund and Marklund.^[28] All calculations were made as per gram fresh weight.

Estimation of anti-estrogenic property

Healthy virgin female mice, Swiss albino, were divided into five groups ($n = 6$). One group was dosed with *n*-hexane extract of *B. suffruticosa* whole plant (100 mg/kg body weight) (designated as a control), another group received ethinyl estradiol alone (100 $\mu\text{g}/\text{animal}$), third group received extract, as well as ethinyl estradiol, fourth group was dosed tamoxifen and ethinyl estradiol and fifth group consist of normal animals (designated as an untreated). Anti-estrogenic activity was determined after daily administration of extract and subcutaneous injection of ethinyl estradiol for 7 days. Uterine weight at the end of the experiment was used as a parameter for the anti-estrogenic property.^[29]

Statistical analysis

All data have been represented as a mean \pm standard error of the mean. Data were analyzed using paired *t*-tests within groups and $P < 0.05$ was considered significant. Linear correlation was established between the LPO and SOD values on day 5 (4.40 am) and the correlation coefficient was calculated.

RESULTS AND DISCUSSION

Thin layer chromatography fingerprint profile of *Bergia suffruticosa*

Figure 1a shows the TLC fingerprint profile of the *n*-hexane extract of *B. suffruticosa*. The TLC showed the presence of β -sitosterol and lupeol [Figure 1b] along with several other compounds (unknown) after derivatization. The R_f value of β -sitosterol and lupeol was found to be 0.31 and 0.52, respectively.

Carrageenan induced acute inflammation

The result obtained indicates that the *n*-hexane extract possess significant ($P < 0.05$) anti-inflammatory activity in an acute model of inflammation induced by carrageenan. The *n*-hexane extract at the test dose of 100 mg/kg body weight reduced the edema by 80% at 3 h, whereas the positive control (diclofenac sodium, 10 mg/kg) showed 69.7% of inhibition [Table 1].

Formalin induced chronic inflammation

The results obtained indicate that *n*-hexane extract significantly ($P < 0.05$) reduced the paw edema induced by formalin in rats. The *n*-hexane extract at the dose 100 mg/kg body weight reduced the edema by 75% on 5th day, whereas the positive control (dexamethasone, 5 mg/kg) showed 82.5% of inhibition when compared to the disease control group [Table 2].

Anti-implantation activity

Control animals showed implantation sites (48 sites/6 animals), whereas animals treated with *n*-hexane extract of *B. suffruticosa* for 6 days of pregnancy, showed complete inhibition in blastocyst implantation [Figure 2].

Estimation of lipid peroxidation and assay of superoxide dismutase enzyme activity

SOD and superoxide anion radical levels (measured as MDA levels) were measured in the uterus of control and treated animals, from days 1 to 6 of pregnancy. Control animals showed a sharp decrease in SOD levels and a corresponding increase in superoxide anion radicals at the time of implantation (day 5, 4:40 am, $P < 0.05$) when compared to days

4 and 5 (10:00 am) of pregnancy. A negative correlation was observed ($r = -0.902$) between the levels of superoxide anion radical and SOD in the uterus at the time of blastocyst implantation [Figure 3].

However, in the *n*-hexane extract treated animals, levels of superoxide anion radical and SOD were altered during the blastocyst implantation. A sharp decrease in superoxide anion radical and an increase in SOD activity was observed ($r = 0.1570$) when compared to days 4 and 5 (10:00 am) of pregnancy [Figure 4]. *n*-Hexane extract reduced elevated MDA level from 6 to 2.5 nmol/g $\times 10^{-5}$ and increased SOD enzyme activity from 0 to 350 units/g in treated animals on the 5th day of pregnancy. This difference in superoxide anion radical and SOD activity between control and *n*-hexane treated animals are statistically significant ($P < 0.05$).

Anti-estrogenic property

Animals treated with the *n*-hexane extract and ethinyl estradiol (both) exhibited a decrease in uterine weight as compared to animal treated with ethinyl estradiol alone ($P < 0.05$) [Figure 5]. Extract decreased uterine weight from 0.33 to 0.2 g in estradiol treated animals, which suggest the anti-estrogenic property of the *n*-hexane extract of *B. suffruticosa*. Moreover, this result is comparable with positive control tamoxifen, which reduced uterine weight from 0.33 to 0.22 g.

TLC fingerprint profile of *n*-hexane extract of *B. suffruticosa* clearly shows the presence of β -sitosterol and lupeol. Our previous phytochemical study also showed the presence of gallic acid, gallicin in the plant.^[8] These compounds have been reported to have anti-inflammatory activity. However, to the best of our knowledge anti-inflammatory activity is not reported for this plant till yet. Pharmacological evaluation of *n*-hexane extract was carried out to explore the anti-inflammatory and anti-implantation activity of this plant.

Table 1: Percentage edema protection by *n*-hexane extract of *Bergia suffruticosa* whole plant in carrageenan-induced acute inflammation

Drug	Dose (mg/kg)	Difference in paw volume after 3 h (mL)	Protection (%)
Positive control (diclofenac sodium)	10	0.259 \pm 0.006	66.7
<i>Bergia suffruticosa</i>	100	0.166 \pm 0.013	80.0
Diseased control	-	0.83 \pm 0.0439	-

Values are expressed as a mean \pm SEM; ($n=6$), $P < 0.001$. SEM: Standard error of mean

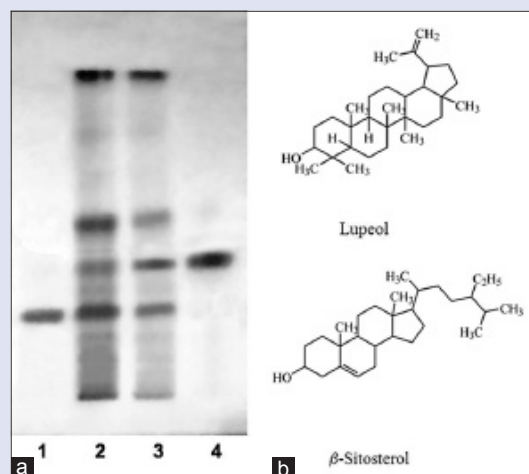


Figure 1: (a) Thin layer chromatography fingerprint profile of *n*-hexane extract of *Bergia suffruticosa* whole plant. (1) β -Sitosterol standard. (2 and 3) *n*-Hexane extract (4) lupeol standard. (b) Structure of lupeol and β -sitosterol

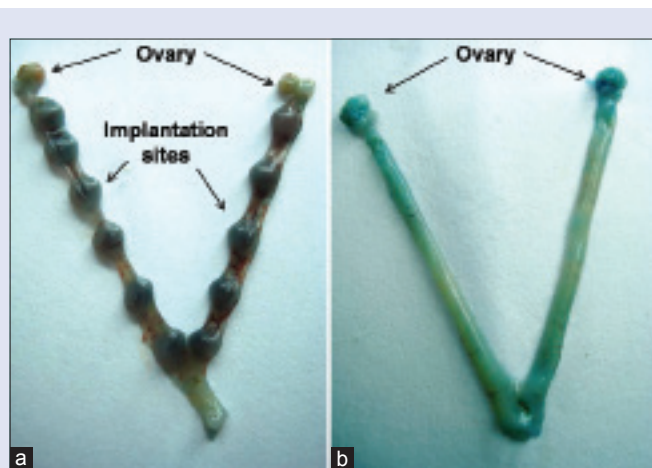


Figure 2: Uterus of (a) Control mice showing blastocyst implantation sites (b) *n*-hexane extract of *Bergia suffruticosa* treated mice, not showing any implantation site

The carrageenan induced paw edema model is generally used to evaluate the acute anti-inflammatory potential of natural products. Carrageenan induced edema formation consist of two phases, that is, an early or first phase (0–2.5 h after carrageenan injection) and a late or second phase (6–12 h postinjection). The early phase results from the release of inflammatory mediators such as serotonin, histamine, and bradykinins, whereas the late phase is associated with the release of PGs.^[30] The *n*-hexane extract at the test dose of 100 mg/kg body weight reduced the edema by 80% after 3 h which suggest the inhibitory activity on the release of prostaglandins.

Chronic inflammation is produced when the body fails to respond against anti-inflammatory agents that further leads to fibroblast proliferation and formation of granulomatous tissues.^[31] However, the results of this study inferred that *n*-hexane extract (~75%) showed potent chronic anti-inflammatory activity, which was comparable to dexamethasone (82.5%). The inflammatory process is reported to be associated with the generation of reactive oxygen species.^[31] The anti-inflammatory activity of this fraction may be attributed to their free radical scavenging activity, which may be attributed to the presence of β -sitosterol and lupeol.

The process of blastocyst implantation is considered analogous to a “pro-inflammatory” response. Therefore, the participation of various inflammatory mediators is contemplated in the process of implantation. *n*-Hexane extract inhibited the blastocyst implantation in the uterus as evidenced by the absence of implantation sites. These results showed that implantation failure cannot be due to the interference with the tubal transport of the fertilized egg. This phenomenon can be attributed to the hostile environment of the endometrium because of the *n*-hexane extract. The preimplantation and peri-implantation periods of the embryo and

the endometrium include a number of biochemical, biophysical, and molecular changes in the endometrial and blastocyst membrane.^[24] The successful completion of these events involves a complex series of synchronized changes in the blastocyst and the endometrium. Interaction between the blastocyst and the endometrium is mediated by number of signals generated by molecules such as cytokines, growth factors, free radicals, and adhesion molecules which are produced and/or secreted by the endometrium and blastocyst.^[18,19] Membrane fluidity, a prerequisite for implantation is achieved by all the above mediators. It is critical that the fertilized eggs reach the uterus at an appropriate proggestational stage of the ovarian cycle and when the endometrium has reached a precise stage of maturity. In mouse, the entry of eggs into the uterus is timed to coincide with the beginning of the luteal phase, which occurs 3 days after fertilization.^[32,33] The normal 3 day stay of the eggs in the uterus is largely governed by an appropriate level of progesterone and estrogen that controls their survival and implantation.^[33]

The arrival of the zygote in the endometrium is insufficient to ensure implantation; hormone-dependent changes and the increase in membrane fluidity also called “receptive endometrium,” are required for successful blastocyst implantation. Superoxide anion radical surge at the time of implantation has been implicated for induction of endometrial membrane fluidity (receptive endometrium). Hence, SOD and superoxide anion radical were used as a marker for blastocyst implantation.^[23,34] Estrogen surge at the time of implantation has been shown to be responsible for the decrease in SOD levels and increase in superoxide anion radical levels (as shown in the control animals).^[32] However, this pattern was altered in the *n*-hexane extract treated animals were SOD levels increase and radical levels fall significantly at the time of implantation. Membrane fluidity, a prerequisite for implantation, is achieved by high free radical levels

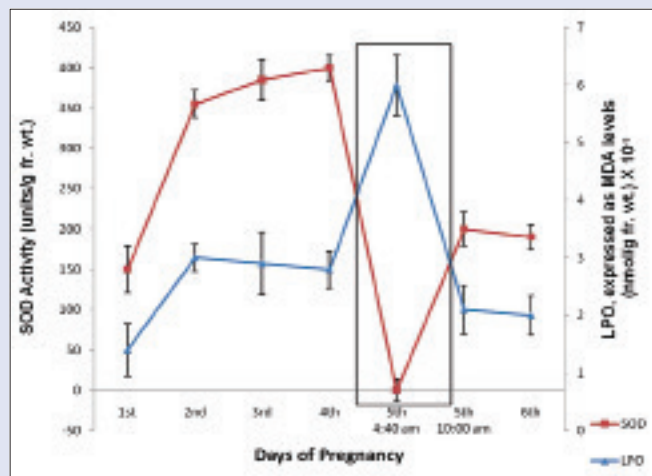


Figure 3: Superoxide dismutase activity and lipid peroxidation (lipid peroxidation; malonaldehyde levels) on different days of pregnancy (days 1–6) in uterus of control animals ($n = 6$) ($r = -0.902$). Selected area shows “window of implantation”

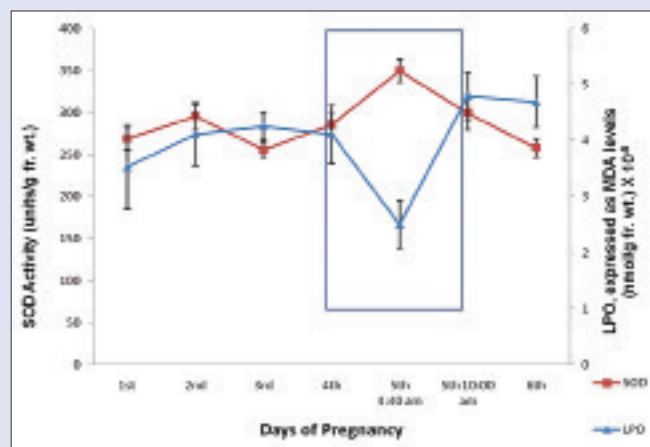


Figure 4: Superoxide dismutase activity and lipid peroxidation (lipid peroxidation; malonaldehyde levels) on different days of pregnancy (days 1–6) in uterus of *n*-hexane extract of *Bergia suffruticosa* treated animals ($n = 6$) ($r = 0.157$)

Table 2: Percentage edema protection by *n*-hexane extract of *Bergia suffruticosa* whole plant in formalin induced chronic inflammation

Drug	Dose (mg/kg)	Difference in paw edema volume (mL) (%)				
		Day 1	Day 2	Day 3	Day 4	Day 5
Positive control (dexamethasone)	5	0.08±0.004 (46)	0.20±0.03 (55)	0.20±0.05 (60)	0.09±0.004 (66.7)	0.14±0.03 (82.5)
<i>Bergia suffruticosa</i>	100	0.07±0.002 (55)	0.18±0.03 (60)	0.18±0.04 (63)	0.08±0.003 (70)	0.20±0.02 (75)
Diseased control	-	0.15±0.02	0.45±0.04	0.50±0.04	0.27±0.03	0.80±0.04

Values are expressed as a mean±SEM, ($n=6$), $P<0.001$. SEM: Standard error of mean

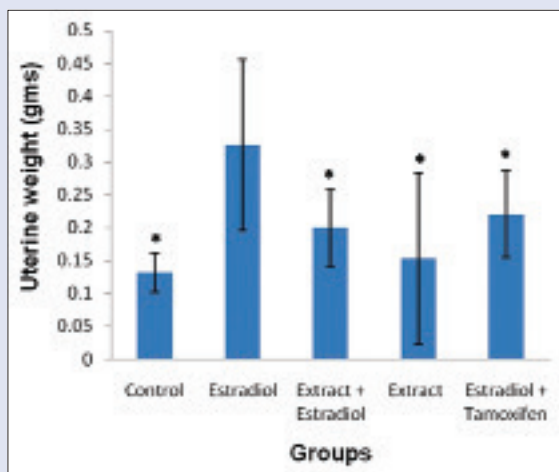


Figure 5: Uterine weight in control and different groups of treated animals ($n = 6$). * $P < 0.05$ was considered significant when compared to ethinyl estradiol alone treated animals

and the treated animals showed low superoxide anion radical levels, thus, resulted in a “nonreceptive endometrium.” In mouse, the estrogen surge occurs on day 4 of pregnancy, approximately 15–25 h prior to implantation.^[33] The *n*-hexane extract showed potent anti-estrogenic activity, which was comparable with tamoxifen. Tamoxifen is a selective estrogen receptor modulator. It inhibits blastocyst implantation when administered postcoitally and reduces LPO,^[35,36] accomplished rationale for its selection in this study. Hence being comparable with tamoxifen, *n*-hexane extract might be interfering with uterine estrogen utilization, exerting anti-estrogenic influence. The observed acute and chronic anti-inflammatory activity of *n*-hexane extract might be due to down-regulation of various mediators of inflammatory cascade such as COX, various cytokines and NO at the time of implantation. Hence, extract modulates two different yet interrelated pathways; inflammatory cascade and estrogen signaling, resulted in altered physiology of the endometrium that is, changing the oxyradical and antioxidant status, making it “nonreceptive” which might have led to failure of blastocyst implantation.

CONCLUSION

n-Hexane extract of *B. suffruticosa* exhibited good anti-inflammatory activity in acute and chronic models of inflammation. *n*-Hexane extract also showed the good anti-estrogenic property. Anti-implantation activity of *n*-hexane extract is attributed to its anti-inflammatory and anti-estrogenic potential. β -Sitosterol and lupeol along with other components of *n*-hexane extract of *B. suffruticosa* might have affected the multiple inflammatory pathways, as well as estrogen signaling pathway thus leading to physiological changes in the endometrium and subsequently in failure of implantation. Further work is in progress to identify the chemical compounds responsible for the observed anti-implantation activity.

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Conflicts of interest

Authors declare that there are no conflicts of interest.

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