SHORT REPORT

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Synthesis and Blastocyst Implantation Inhibition Potential of Lupeol Derivatives in Female Mice

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Abstract: Blastocyst implantation which is analogous to pro-inflammatory response, mediated by different inflammatory mediators and ovarian hormones found to be an effective target for the development of emergency contraceptives. In the present study, a series of derivatives an anti-inflammatory natural scaffold, lupeol were synthesized under mild reaction conditions and good yield. All the compounds were evaluated for acute anti-inflammation. The three active compounds with 62-92% edema protection were screened for chronic anti-inflammation. The analogue 3-(p-chlorocinnamoyl) lupeol (**2)** with potent anti-inflammatory activity (85% protection) was evaluated for the anti-implantation activity by studying changes in superoxide dismutase (SOD) and lipid peroxidation (LPO) levels, visualization of implantation site and anti-estrogenic activity. As expected, a sharp decrease in superoxide anion radical and increase in SOD activity was seen in the endometrium of treated animals. Also no implantation sites were observed in the uterus of treated animals. The active compound also exhibited anti-estrogenic activity.

Keywords: Lupeol; contraceptive; anti-inflammatory; blastocyst implantation; anti-estrogenic.

1. Plant Source

Emblica officinalis bark was collected from the Daskoi, Gujarat, authenticated and the voucher specimen (NIPER-A/NP/0811/21) was deposited in the Dept. of Natural Products, NIPER-Ahmedabad.

2. Previous Studies

The population of India by March, 2011 was more than 1.21 billion and will be 1.65 billion by 2050 and will take over China [1]. Though several methods of contraception such as hormonal (levonorgestrel), combined hormonal (oestrogen and progestogen) and the selective progesterone receptor antagonist (mifepristone) etc are available, these steroidal drugs cause serious disorders such as hypertension, myocardial infarction, cancer and weight gain [2]. Hence, there is a continuous effort in the development of

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non-steroidal contraceptives from natural sources or natural derived products because of their chemical diversity and biochemical specificity.

Blastocyst implantation, one of the targets for emergency contraception is considered as analogous to pro-inflammatory response, which is modulated by various pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), nitric oxide (NO), superoxide anion radicals, interleukin-1(IL-1), leukemia inhibitory factor (LIF), colony-stimulating factor (CSF) and ovarian hormones. These mediators play an important role in uterine receptivity and blastocyst implantation [3-6]. However, ovarian steroid hormones, estrogen and progesterone regulate all the mediators and hence blastocyst implantation [3]. Prostaglandins (PGs) are also implicated as important mediators for increased endometrial vascular permeability during implantation, which is evident from delayed implantation or implantation failure by COX-1 and COX-2 inhibitors [7]. The molecular signals that regulate the blastocyst implantation are of clinical relevance since understanding the nature of the signals may lead to strategies to correct implantation failure or to develop novel contraceptives.

Pentacyclic triterpenes are reported to exhibit anti-inflammatory activity [8]. Lupeol has been extensively studied for its inhibitory effects on inflammation [9, 10], anti-arthritic [11] and antioxidant activity [12] etc. In continuation of our research on development of nonsteroid, novel, potent emergency contraceptive agents, we synthesised derivatives of lupeol and evaluated for *in vivo* acute and chronic anti-inflammatory, anti-implantation as well as anti-estrogenic activity.

3. Present Study

*Isolation of lupeol (**1**) and Synthesis of derivatives:* The *E. officinalis* bark powder (800 g) was extracted with *n*-Hexane in a soxhlet apparatus. The extract (21g) was subjected to column chromatography and lupeol was obtained using *n*-hexane to increase polarity of ethyl acetate in hexane as eluting solvent with yield 1.1% and identified by physical and spectral analysis, which was found to exactly matching with the reported values [13].

To a solution of compound **1** in dichloromethane, N,N-dicyclohexyl carbodiimide and N,N-dimethyl amino pyridine was added followed by appropriate cinnamic acids. The mixture was sonicated for 15 min. The reaction mixture was filtered, concentrated and purified by column chromatography to obtain the corresponding cinnamic acid esters 3-O-(p-chlorocinnamoyl) lupeol (**2**, 78%) and 3-O-cinnamoyl lupeol (**3**, 91%). To the mixture of **1,** acetic anhydride was added with a drop of pyridine and kept overnight at room temperature. The usual work up and crystallization yielded lupeol acetate (**4**, 85%).

Room temperature stirring of **1** in dichloromethane and pyridinium chlorochromate with silica gel (1:1) for 4.5 h and filtration yielded compound **5** (90%). Compound **5** in dichloromethane was stirred with hydroxylamine hydrochloride in presence of pyridine at room temperature for 6 h and usual work up resulted compound **6** (80%). A mixture of compound **5**, phenylhydrazine and glacial acetic acid was refluxed under N_2 for 1 h distilled water was added and extracted with dichloromethane. The dichloromethane extract was washed with 5% aqs. NaOH and brine, dried over anh. Na_2SO_4 and concentrated under vacuum to afford pale yellow solid. The product obtained was purified by column chromatography to obtain compound **7** (56%).

Compound **5** in DCM was stirred with ethyl formate in presence of NaOMe under N_2 for 4 hr Ice-water was added and extracted with ethyl acetate, dried over anh. Na_2SO_4, filtered, concentrated and crystallized to obtain compound **8**(61%). Compound **5** in dichloromethane and conc. HCl was treated with $NaNO_2$ in water at 0˚C. The reaction was quenched with ice, extracted with dichloromethane and crystallized to get compound **9** (75%) (Scheme1). All the compounds were identified by analytical and spectral data analysis (Supporting information).

Animals: The experimental protocols were approved by the Institutional Animal House Ethics Committee (IAEC), constituted by the Ministry of Social Justice and Empowerment, Government of India. Anti-inflammatory studies were carried out using healthy Sprague Dawley rats of either sex, weighing between 150-250 g. Animals were housed 4 per cage under the environmental condition of 10% air exhaust in the AC unit, relative humidity of 60±5%, 12:12 h day-night cycles at a temperature of 24±3˚C. Animals were divided into 4 groups of 6 each and put on fasting for 12-18 h prior to experiment. One group per compound was taken for experiment. Mature, female mice (Swiss Albino strain, Mus musculus, 2-3 months old) were used for the anti-implantation activity.

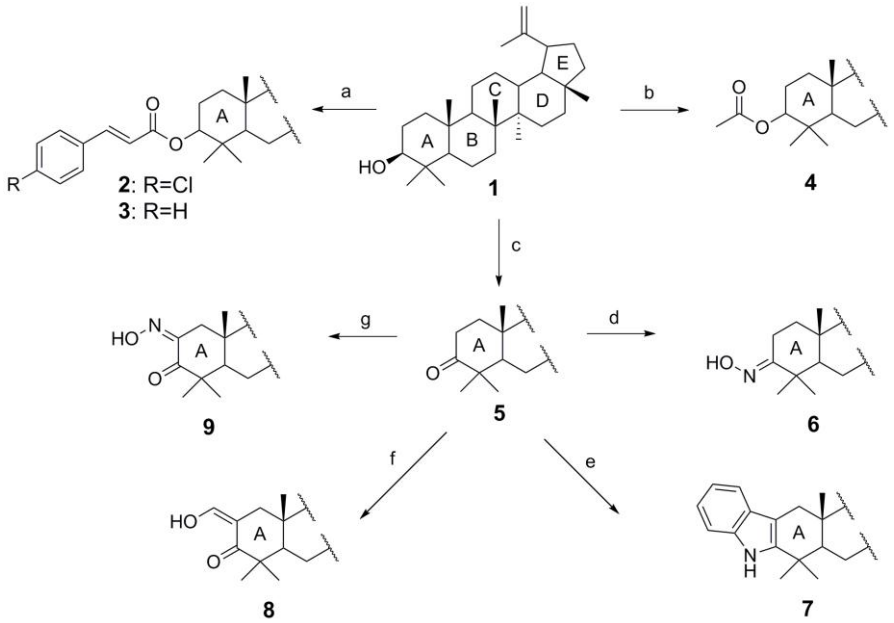

Figure 1. Synthesis of different derivatives of compound **1**: Reagents and conditions: **a**) DCC, DMAP, corresponding cinnamic acids, sonication, 15 min; **b**) $(CH_3CO)_2O$, Py, 24 h; **c**) PCC, SiO_2, DCM, rt, stirring, 4.5 h; **d**) $NH_2OH.HCl$, Py, rt, stirring, 6 h; **e**) Phenyl hydrazine, CH_3COOH, reflux under N_2, 1h; **f**) Ethyl formate, NaOMe, DCM, stirring under N_2, 4 h; **g**) $NaNO_2$, conc. HCl, 0^oC.

Acute inflammation: Animals were dosed with diclofenac sodium (10 mg/kg) or test compounds (50 mg/kg) 1 h before injection. All the compounds were orally administered with 0.2% agar suspension freshly prepared as a vehicle. Carrageenan solution (0.1 mL of 1% solution) was injected in the sub-plantar 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 up to 3h. Inhibition of (%)edema was calculated.

Derivatives **2**, **4** and **9** along with the parent compound (**1**) significantly exhibited strong acute anti-inflammatory activity with 60-93% protection compared to diclofenac sodium (69%). The introduction of p-chlorocinnamic moiety at C-3 (**2**) exhibited better activity (93%) than the parent compound **1** (70%) and positive control. The 3-acetate compound (**4**) showed 66% followed by derivative **9** with 62% protection (Table 1, Supporting information).

Chronic inflammation: Animals were dosed with Dexamethasone (5 mg/kg) or test compounds (50 mg/kg) 1 h before the injection. All compounds 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 hr reading. Formalin solution (0.1 mL of 2% solution, prepared in normal saline) was injected in the sub-plantar region of the right hind paw of rats 1h after drug administration. Final hour reading was recorded 3h after the injection. This exercise was continued for five consecutive days. Degree of inflammation was measured on all five days. The inflammation was assessed by calculating the % protection.

Compounds, **1**, **2** and **4** exhibited very good chronic anti-inflammation activity with protection of 69-85% up to 5^{th} day and the derivative **2** was more active with 85% protection than the drug dexamethasone (83%) (Table 1, Supporting information).

Anti-implantation activity: 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 by Caligioni *et al*. [14]. Females that showed a proestrus smear (Day 0) were mated with a male of proven fertility on the same evening. Presence of a vaginal plug (next day morning) confirmed mating and was designated as Day 1 of pregnancy. Animals were divided into two groups, A and B. Group A and B were dosed orally at 10:00 a.m. each day from day 1 to day 6 of pregnancy with 0.2% agar (control) and the active analogue (50 mg/kg), respectively. The pregnant females from both the groups were sacrificed on 1, 2, 3, 4, 5 day (4:40 a.m.), 5 day (10:00 am) and 6 day of pregnancy. The uterus was excised, cleaned from adhering fat, washed with normal saline, weighed and used for estimation of lipid peroxidation and assay of superoxide dismutase (SOD) activity.

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. The homogenate was centrifuged at 3500 rpm for 10 minutes. The pellet was resuspended in 0.1 mL of HBSS and used for estimation of lipid peroxidation. Lipid peroxidation was measured in terms of malonaldehyde (MDA):thiobarbituric acid (TBA) reaction as reported [15]. The reaction mixture was having 0.1 mL of tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid (pH - 3.5 with 1 M NaOH) and 1.5 mL of 0.8% aqueous solution of TBA. Reaction mixture was made to a volume of 4 mL with 0.7 mL of double distilled water and heated at 95ºC for 1 h. Then double distilled water (1 mL) and a mixture of *n*-butanol and pyridine (15:1 v/v, 5 mL) was added and the mixture was shaken vigorously in a vortex mixer for 5 min. This mixture was then centrifuged at 3000 rpm for 7 min. The upper organic layer was separated and the amount of MDA formed was measured at 532 nm. Appropriate controls were used at different steps during this estimation (extinction coefficient of MDA=1.45 x 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 (3x 30 sec). The homogenate was treated with 1 mL of 0.1% Triton X 100 (v/v) for 20 min at 4ºC. Homogenate was then centrifuged at 10,000 rpm at 4ºC for 30 min. The supernatant was used for the assay of superoxide dismutase (SOD) activity by the method as reported by Marklund and Marklund [16]. Briefly, Supernatant was transferred to both plastic tubes and glass tubes in 2:3 ratios. Plastic-tubes containing enzyme were kept at 4°C and glass tubes were kept in boiling water bath for 45 min. Then, boiled content was centrifuged at 5000 rpm for 10 min at 25˚C. Absorbance were taken for both enzyme and boiled samples at 420 nm. All calculations were made as per gram fresh tissue weight.

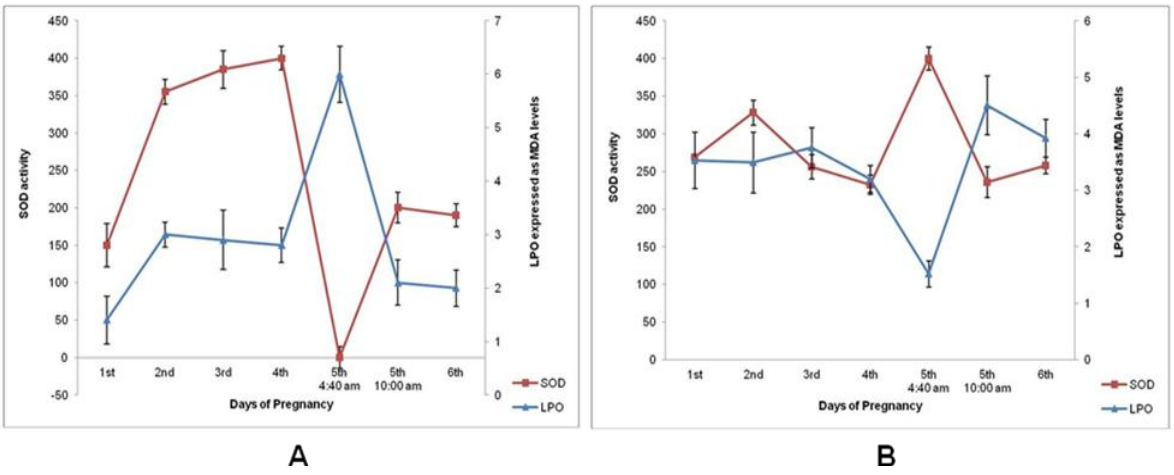

Figure 2. A: SOD (unit/gram fresh weight) and LPO expressed as MDA levels, (nmol/gram fresh weight) levels on day 1-6 in uterus of control animals (n=6). **B**: SOD and LPO levels in uterus of compound **2** treated animals (n = 6).

The result reveals that control animals show a sharp decrease in SOD levels and an increase in superoxide anion radical at the time of implantation (day 5 at 4.40 am, Fig.1A). However, these levels were altered in animals treated with derivative **2** which showed an increase in SOD activity and decrease in superoxide anion radical levels (Fig.1B) which is one of the requirements for inhibiting blastocyst implantation. The changes in superoxide anion radical and SOD activity between control and treated animals are statistically significant (p<0.05).

Visualization of implantation site: Another set of animals (n=6) from each group were injected intravenously 0.1 mL of 1% Chicago blue 6B dye through tail vein on day 7 of pregnancy. All the animals were sacrificed and the uterus was exposed to count the number of implantation sites. The uterus of control animals showed (Fig. 2A) blastocyst implantation sites(~10 nos.) in each, while the derivative **2** completely inhibited the blastocyst implantation (100%) in the treated animals (Fig. 2B). The alteration of SOD and LPO levels were further confirmed by the inhibition of blastocyst implantation in animals treated with derivative **2**.

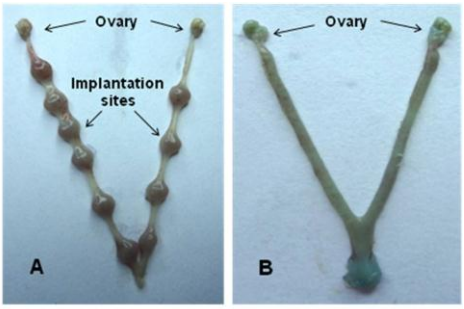

Figure 3. A. Blastocyst implantation in uterus of control animals; **B.** No implantation in uterus of treated animals.

Estimation of anti-estrogenic activity: Healthy virgin female mice, Swiss Albino, were divided into six groups (n=6). First group received 0.2% agar solution (control), second group received tamoxifen (0.063 mg/kg, positive control), third group received ethinyl estradiol (EE,100 µg/animal), fourth group received EE and tamoxifen, fifth group received the active analogue **2** (50 mg/kg) and sixth group received EE and the active analogue **2**. Anti-estrogenic activity was determined after daily oral administration of drug and subcutaneous injection of EE for 7 days. Uterine weight at the end of the experiment was used as a parameter for anti-estrogenic property [17]. The uterine weight of animals treated with only 3-p-chlorocinnamoyl derivative (**2)** and **2** along with ethinyl estradiol (EE) found to decrease as compared to animals treated with EE alone (p<0.05), which suggest the anti-estrogenic property of the derivative **2** (Fig. 3) which is significant in comparison with positive control tamoxifen.

Statistical analysis: All data have been represented as mean ± SEM. 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 a.m.) and the correlation coefficient was calculated.

The hormone-dependent changes and the increase in membrane fluidity, which makes the endometrium receptive is necessary for successful blastocyst implantation[18]. The results indicate that failure in implantation is not due to the interference in tubal transport but the unfavourable environment generated by the derivative **2**. As 3-(p-chlorocinnamoyl) lupeol(**2**) showed anti-estrogenic property, it might be interfering in uterine estrogen utilization there by change in the biophysical and biochemical events. Moreover, it also possess potent acute and chronic anti-inflammatory activity, hence it might have down regulated some of the inflammatory mediators such as cyclooxygenase, cytokines, nitric oxide and superoxide anion radicals at the time of implantation. Hence, 3-(p-chlorocinnamoyl) lupeol (**2**) modulates two different interrelated pathways i.e. inflammatory cascade and estrogen signalling, resulting in altered physiology of the endometrium. Consequently, it can be a good candidate for assessment of molecular mechanism involved in the implantation failure and developed as a potential contraceptive agent.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

References

[1] http://censusindia.gov.in/. Accessed on Jan. 29, 2011
[2] O. Blashkova, A. Sasko, A. Pontuch, T. Veselska, A. Lipovska and I. Kostolny (1981). Side effects of oral contraceptives, *Bratisl Lek Listy.* **75(2)**, 167-172.
[3] J. S. Hunt, L. Miller, K. F. Roby, J. Huang, J. S. Platt and B. L. DeBrot (1997). Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes, *J. Reprod. Immunol.* **35**, 87-99.
[4] M. Laloraya, P. G. Kumar and M. M. Laloraya, (1989). Possible role of superoxide anion radical in the process of blastocyst implantation in *Mus musculus*, *Biochem. Biophy. Res. Comm.* **161(2)**, 762-770.
[5] A. Lindhard, U. Bentin-Ley, V. Vibeke Ravn, H. lslin, T. Hviid, S. Rex, S. Bangsboll and S. Sorensen,

(2002). Biochemical evaluation of endometrial function at the time of implantation, *Fertil. Steril*. **78(2)**, 221-233.

[6] G. Yulia, G. Irit, B. A. Paulomi, B. Amihai, O. Yuval, S. Edna, M. Gil and D. Nava (2010). Local injury of the endometrium induces an inflammatory response that promotes successful implantation, *Fertil. Steril*. **94**, 2030-2036.

[7] P. L. Pakrasi and A. K. Jain (2007). Effect of cyclooxygenase on "window of implantation" in mouse, *Prost. Leuko. Essen. Fatty Acids*. **77**, 147-153.

[8] B. De las Heras, B. Rodríguez, L. Boscá and A. M. Villar (2003). Terpenoids: sources, structure elucidation and therapeutic potential in inflammation, *Curr. Top. Med. Chem.* **3**, 171-185.

[9] M. Saleem (2000). Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene, *Cancer Lett.* **285**, 109-115.

[10] M. A. Fernandez, B. De las Heras, M. D. Garcia, M. T. Saenz and A.M. Villar (2001). New insights into the mechanism of action of the anti-inflammatory triterpene lupeol, *J. Pharm. Pharmacol.* **53**, 1533-1539.

[11] R. B. Agarwal and V. D. Rangari (2003). Anti-Inflammatory and anti-arthritic activities of lupeol and 19α–H lupeol isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* roots, *Ind. J. Pharmacol.* **35**, 384–387.

[12] M. Nagaraj, S. Sunitha and P. Varalakshmi (2000). Effect of lupeol, a pentacyclic triterpene on the lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure, *J. Appl. Toxicol.* **20**, 413–417.

[13] Sukh Dev., A. S. Gupta and S. A. Patwardhan, In CRC Hand Book of Terpenoides, vol. II, CRC Press, Inc. Boca Raton, FL. (1989).

[14] C. S. Caligioni (2009). Assessing reproductive status/stages in Mice, *Curr. Proto. Neurosci.* **48**, A.4I.1-A.4I.8.

[15] H. Okahwa, N. Ohishi and K. Yagi (1979). Reaction of linoleic acid hydroperoxides with thiobarbituric acid, *Anal. Biochem.* **95**, 351-358.

[16] S. Marklund and G. Marklund (1974). Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.* **47**, 469-474.

[17] S. P. Hiremath and H. Rao (1990). Anti-fertility efficacy of the plant *Striga lutea* (Scrophulariace) on rats, *Contraception.* **42**, 467-477.

[18] M. Nivsarkar, M. Patel, H. Padh, C. Bapu and N. Shrivastava (2005). Blastocyst implantation failure in mice due to "non receptive endometrium": Endometrial alterations by *Hibiscus rosa-sinensis* leaf extract, *Contraception.* **7**, 227-230.