

Original Research

Protective effect of Honokiol against endometriosis in Rats via attenuating Survivin and Bcl-2: A mechanistic study

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Abstract: Nearly 10-15% of women in the reproductive age were affected by endometriosis and currently facing the unmet need of effective therapeutic interventions for its management. Concerning this, the present study was intended to investigate the effect of Honokiol (HK) for the treatment of endometrial hyperplasia. The rat endometrial model was established and subsequently administered with a numerous dose of HK. The histopathology of tissues was also investigated. Results showed that, HK effectively inhibit the proliferation of rat edometriotic tissues in a dose dependent manner. In terminal deoxynucleotidyl transferase (TdT) -mediated dUTP biotin nick end labeling (TUNEL) method, HK was able to bring apoptosis in endometrotic cells. Moreover, it also inhibits the mRNA levels of Survivin gene and Bcl-2 (B-cell lymphoma 2) in qPCR and Western blot analysis together with increases the mRNA level of apoptosis promoting factor Bax. These results clearly suggest that, HK was proficient to provoke apoptosis of rat endometriotic cells.

Key words: Honokiol, apoptosis, Bcl-2, Survivin, endometriosis.

Introduction

Nearly 10-15% of women in the reproductive age were affected by endometriosis. It is deemed as a hormone-dependent benign disease which could be easily diagnosed with by the existence of functional endometrium outside of the uterine cavity. Despite of the availability of extensive literature about it, the pathogenesis of the disease is still not realized (1-3). It was suggested long back in 1927 by Sampson that, pathogenesis of endometriosis is retrograde menstruation, but even so it's a matter of disputation. However, retrograde menstruation is a condition when menstrual blood carrying the endometrial cells returns back through the Fallopian tubes and into the pelvic cavity instead of a period. It has been associated with menstrual cramps, loss of fertility, and chronic pelvic pain in the affected women. Various studies also suggest the role of disregulated cellular apoptosis in the endometriosis. It has been well documented that, the normal apoptotic mechanism can able to regulate the usual cellular process for the duration of the menstrual cycle by removing old growing cells from the serviceable layer of uterine edometrium without triggering inflammatory response (4,5).

The oncogenic protein Bcl-2 is a well recognized role in cell suicide program that is vital for development, homeostasis and cellular immunity. The literature survey reveals that under normal apoptotic activity, the level of Bcl-2 has not been very much higher (6). Whereas, in the case of endometriosis, its expression has been continuously upregulated suggesting its role as an important biomarker indicating an alteration in the normal process (7,8). On the other hand, growing evidences suggests that, Survivin, a key bifunctional protein that act as chief regulator of mitosis and suppressor of apoptosis plays an important role in the progression of endometriosis (9-11). Thus, regulation of these two proteins by a specific inhibitor offers various advantages and able to improve the condition of apoptosis.

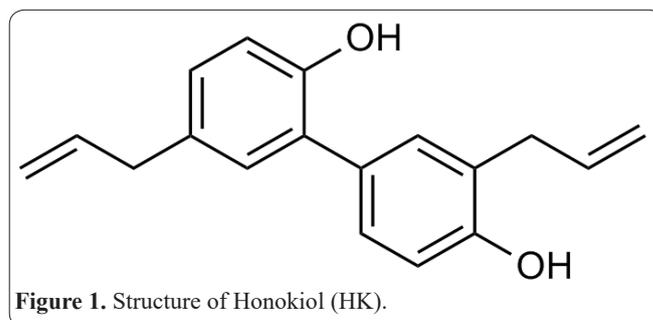


Figure 1. Structure of Honokiol (HK).

Honokiol (HK), a small molecule polyphenol obtained from the genus *Magnolia* is well known for its effect on Bcl-2 proteins for effective management of cancers, Fig 1 (12,13). However, till now no single study has reported the effect of HK on endometriosis. Consequently, encouraged by the above reports, the present study was intended to elucidate the effect and mechanism of HK in endometriosis and the results of the study is the subject matter of the investigation.

Materials and Methods

Animals and materials

From the central animal facility of the Institute, sixty female Sprague – Dawley rats (180 – 200 g) were obtained for the experimental purpose after the approval of Institutional Ethical Committee. The 2X Maxima SYBR Green/ROX qPCR Master Mix Kit, were procured from Fermentas; while, the Indomethacin was obtained from a commercial supplier including all other chemical and reagents.

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The rat endometriosis model

The endometriosis in the rat has been induced in accordance with the established reported procedure given elsewhere (14). Briefly, the selected sixty rats were categorized into two groups. Among which, one group of 10 rats served as sham-operated, group 1, while the next group constitutes the remaining rats, i.e., 50 rats. In a sham treated group, the skin of these rats was sterilized and an incision was made around 3-cm midline in the lower abdomen. The resulting cavity was sutured. The next group containing 50 rats was used for the induction of endometriosis. After a similar incision in the lower abdomen; the left uterine horn was located, where, one side was kept approximately 1 cm from the proximal part of the left uterine and the other side was at about 1 cm from the ovary. The resultant excised tissue was kept in sterile saline. The subway between the muscle of the abdomen and subcutaneous fascia was squeezed to accommodate the uterus and finally sutured after installing the endometrial tissue in the base of the right side of the channel. To prevent any infection at the site, the each rat was administered penicillin for 3 days with 400 000 Units. Later, the operated rats were fed *ad libitum* after 48 h of surgery and were injected with estradiol (0.1 mg/kg/day) for next consecutive four days (thrice a day). The rats were subsequently examined for the growth of ectopic endometrium after surgery.

The rats of the second group after development of endometriosis were divided randomly into five groups, viz., model group, Indomethacin group (1.2mg/kg), HK (5 mg/kg), HK (10 mg/kg) and HK (15 mg/kg) where each group contains ten animal. The group was created in order to accommodate different test dose of test compounds. Whereas, the same volume of distilled water was used in sham-operated control group and the model group. The drugs were constantly given for 30 days and after that they were euthanized. The ectopic endometrium was rapidly removed from them for the further examination.

H & E staining

The above excised endometrium was swiftly fixed in formaldehyde (10%) and implanted in paraffin. Its 4 μ m sections were selected for staining with H & E. These stained sections were visualized with the microscope for detection of any irregularity in morphological characters of tissues.

TUNEL assay

Particularly in this assay, the sections of paraffin were washed twice with xylene for 5 min each, and then twice with 100% ethanol and absolute ethanol to make them hydrated. Later, distilled water was used to rinse this and after cleaning, it was further incubated with Triton X-100 (0.2%) in PBS-Tween or 4h. After completion of the desired time and for preventing any interference with endogenous peroxidase, it was again rinsed twice with PBS-Tween 20 and then incubated in H₂O₂ (3%) in PBS for 10 min. It was further incubated in TdT reaction buffer for 2 h at 37 – 40 °C in a humid environment (100%) after cleaning with PBS-Tween 20. At last, section were incubated with streptavidin-HRP (in PBS) at room temperature. After washing, it was further counterstained with Gill's hematoxylin for

30s, rinsed in fresh tap water for 5 min, and dehydrated via using a graded concentration of ethanol. These were later kept on a coverslip with xylene-based mounting medium.

Quantitative PCR (qPCR) analysis

For this, the total RNA from the affected endometrial tissue was processed using TRIzol reagent (Invitrogen, USA). Whereas, 1 μ g of RNA was reversely transcribed into cDNA using the AMV method. Then, qPCR was performed using the 2X Maxima SYBR Green/ROX qPCR master mix kit with the help of real time PCR. The program was initially carried out for 2 min at 95 °C, followed by 40 cycles of 30s each at 95 °C, and subsequent 60s at 60 °C. The results were determined by $\Delta\Delta C_t$ relative quantification analysis.

Western blot analysis

To perform western blot analysis, the total protein was extracted and used for the further analysis. The resultant protein was then boiled in loading buffer and then disassociated to by 8% Tris-glycine gels to afford Bcl-2, survivin, caspase-3 and caspase-9. The western blotting was performed using mouse anti-rat antibody (Abcam Biotech, USA) and goat anti-mouse secondary antibody. Whereas, the immunocomplexes were visualized by enhanced chemiluminescence.

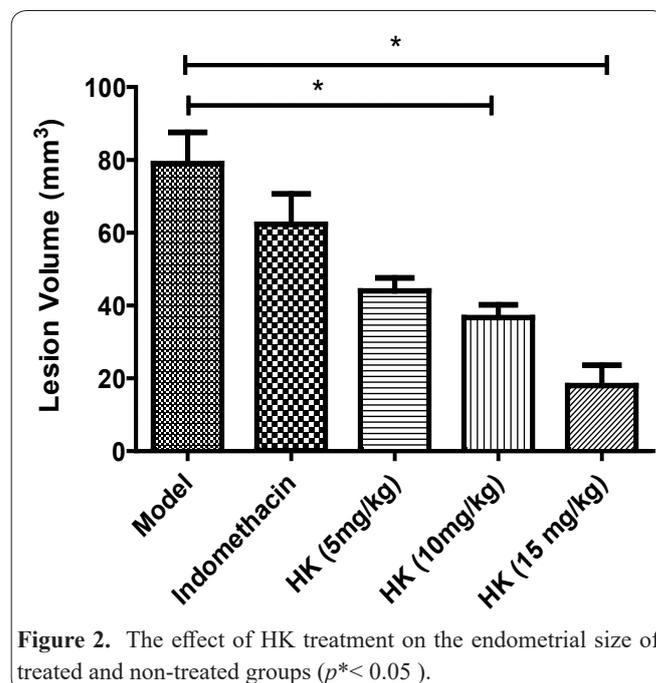
Statistical analysis

For statistical analysis, ANOVA was used and the significance level was set at $p < 0.05$, and $p < 0.01$ suggesting a strongly significant difference. All statistical analysis was performed with the help of Graphpad Prism software (Graphpad Software, Inc, San Diego, USA).

Results

Effect on growth of ectopic endometrium

The effect of test compounds was elucidated in the fig. 2. It has been found that, HK significantly inhibit the progression of endometriosis in a dose-dependent manner more prominently than Indomethacin. On compa-



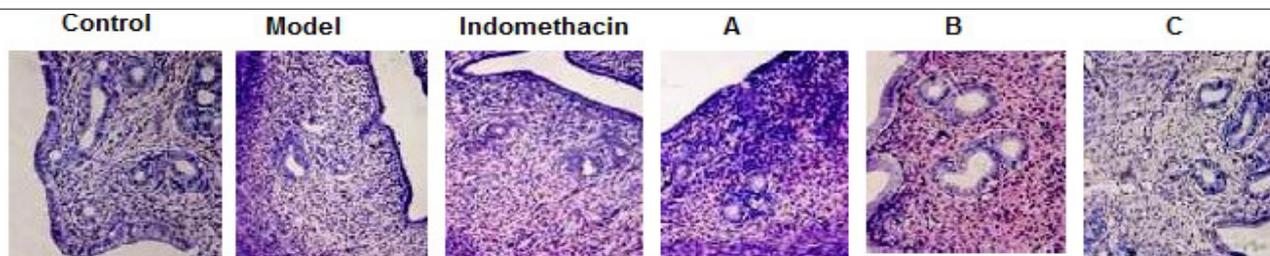


Figure 3. Determination of effect of HK on different treated group on the ectopic endometrial tissue (200× magnification), where A: HK(5mg/kg); B: HK(10mg/kg);C: HK(15mg/kg).

ring the results with the model group, these inhibitory activities were proved as much efficient in controlling the proliferation of the endometriotic tissues in subjects.

Assesment of morphological character of ectopic endometrial tissue

After determination of effect of HK on endometrial tissue, it is worthwhile to perform its histopathological examination using H & E staining. As shown in shown in Fig. 3, the control group does not exhibit any obvious irregularity in the morphological character of the tissues. They are well stratified by simple columnar epithelium which contain higher concentrations of glands. Whereas, the tissues in model group showed the formation of gland cavity-like structure followed by proliferation of epithelial cells. This suggests that, the arrangement was highly disorderd and offered a flase stratified appearance which are now rich in interstitial cells. But after introduction of standard drug indomethacin, these abnormalities were soon reaches to normal level, i.e., the cavity was reduced significantly together with reduction in epithelial cells in comparison to model group. Moreover, the introduction of HK in graded concentration showed similar results by further narrowing the gland cavity associated with reduction in epithelial cells present on its surface.

TUNEL assay of ectopic endometrial tissue

To further confirm these observations, TUNEL labelling was carried out which illustrates that all endometriotic tissues were dispersed with the TUNel-labeled cells, fig. 4, and positive signals were confined in the nucleus showing by green fluorescence. In comparison with the model, the entire treated group demonstrated fluorescence and the positive cells were increased in a concentration-dependent manner. This observation suggests the antiproliferative effect of HK preferably by influencing apoptosis of endometriotic tissues.

Quantitative PCR analysis

In order to determine the underlying mechanism behind this, the next part of the study was aimed to quantify the effect of HK on the transcription levels of Bcl-2, surviving and Bax. It has been found that, treatment of HK considerably suppresses the elevated activity of the above discussed apoptosis-inhibitory factor survivin in a dose-dependent effect, fig. 5. This was further accompanied by inhibition of Bcl-2 and enhancement of apoptosis-promoting factor Bax. It was marked to note that, the ration between Bcl-2/Bax was significantly lessen in response of higher concentration of HK. This study confirms that, HK in graded dose enhanced the activity of Bak, caspase-3 and caspase-9 via upregulation to

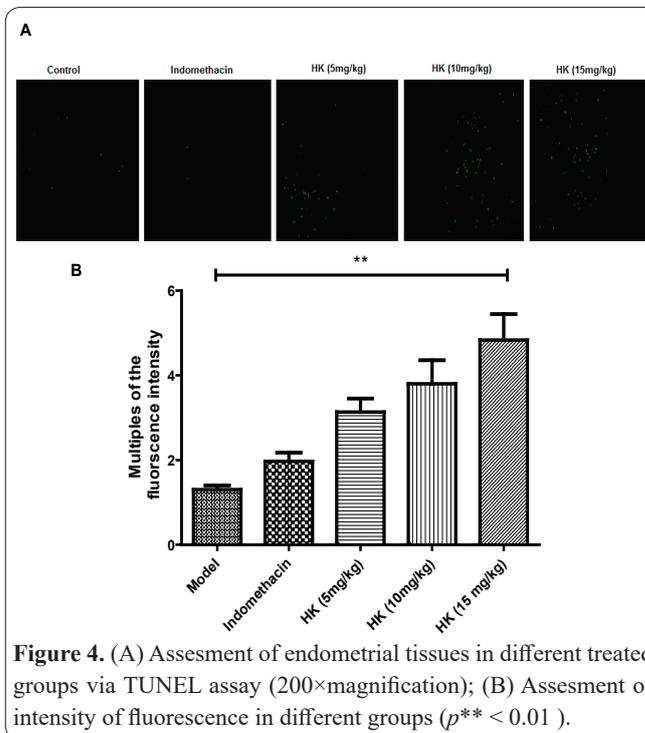


Figure 4. (A) Assesment of endometrial tissues in different treated groups via TUNEL assay (200×magnification); (B) Assesment of intensity of fluorescense in different groups ($p^{**} < 0.01$).

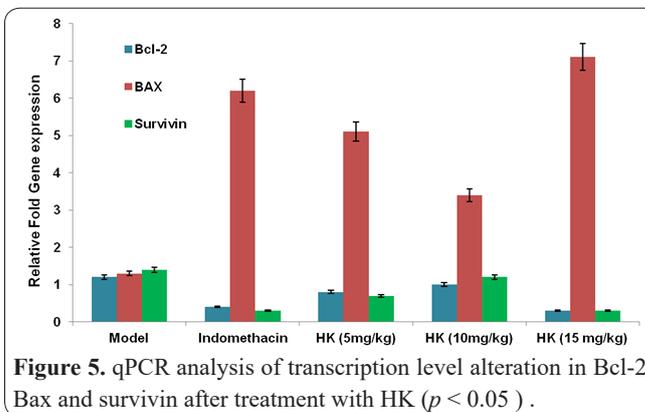


Figure 5. qPCR analysis of transcription level alteration in Bcl-2, Bax and survivin after treatment with HK ($p < 0.05$).

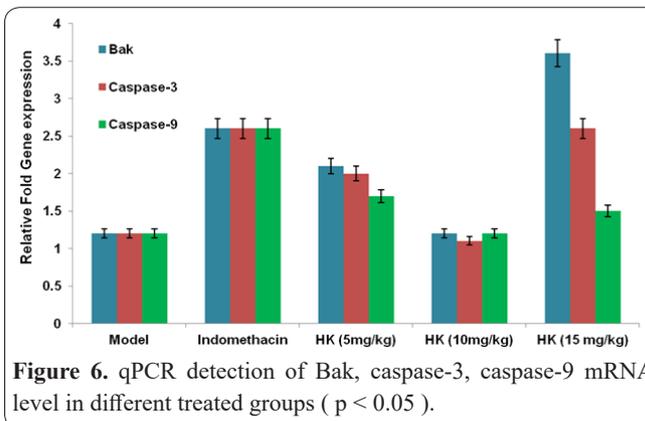
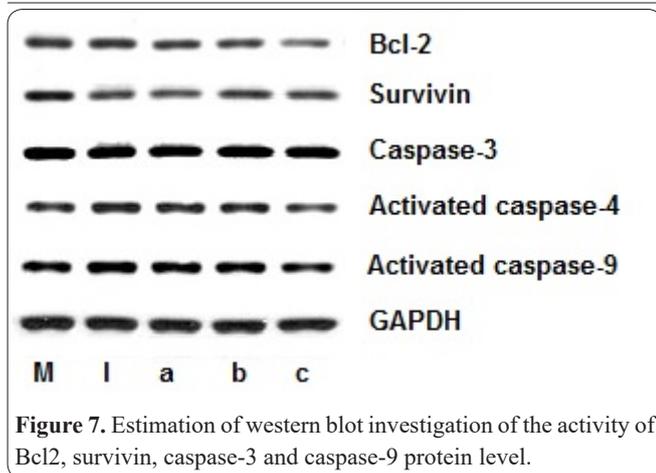


Figure 6. qPCR detection of Bak, caspase-3, caspase-9 mRNA level in different treated groups ($p < 0.05$).

promote apoptosis (fig. 6).

Western blot analysis



The western blot analysis on the levels of Bcl2, survivin, caspase-3 and caspase-9 as shown in fig 7 indicated that, the treatment of HK considerably up-regulate the activity of caspase-3 and -9 translation. Whereas, it causes the down-regulation of Bcl2 and survivin suggesting its role in apoptosis.

Discussion

The present work provides a fresh insight about the protective effect to Honokiol against the edometriosis. This study become more imperative to perform because of the limited clinical interventions for the its effective management. Current therapeutic approaches are mainly based on reducing the level of endogenous steroid production via use of combined oral contraceptive pills, progestins, danazol which provides short-term symptomatic care. However, the clinical utility of these agents has been seriously compromised in the management of endometriosis because of the risk of osteoporosis, strong anti-estrogenic effects, and relapse (15-17). Therefore, the newer agents are needed urgently to fill the unmet medical need.

The HK is well known for its wide array of biological activity with less toxicity. Various in-vivo studies suggests that, it exerts potent anticancer activity mediated via alteration in level of opoptotic proteins. It induces apoptosis with the initiation of Bak, Bax and Bad. Moreover, it also reduces the levels of Bcl-xL and Mcl-1 proteins (12,18). Thus, the present study was undertaken to corroborate the protective of Honokiol against endometriosis because of its above reported activity.

Present study indicated that, HK might be able to significantly attenuate the anti-apoptotic factors of uterine endothelial tissues. In histopathological examination, HK prominently reduces the abnormalities of the tissues in comparison with model group. While, the activities of caspases-3 and -9 were elevated, thereby favoring the apoptosis of uterine endothelial cells. It has been later confirmed with the help of TUNEL assay. Moreover, experiments conducted with the help of qPCR showed that HK can able to lessen the expression of survivin gene which was found highly expressed in the endometrium. Thus, in accordance with the previous studies, administration of HK causes significant reduction in the activity of Bcl-2 and increasing the level of Bax.

As a concluding remark, treatment of HK could be able to protect endometrial cells via attenuation of survivin and Bcl-2 together with promoting a level of Bax.

On the basis of experimental results, it has been suggested that, HK prominently induces apoptosis of endometriotic cells could be used as an effective agent for the management of endometrial hyperplasia.

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