Research Article

Effect of Kitolod (Isotoma longiflora) Extract on Bcl-2 and Caspase-3 Expression in Benzo[a] pyrene-Induced Rat Cervical Tissue

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Abstract

Objective: To evaluate the effect of ethanol extract of *H. longiflora* leaves on apoptosis regulation through Bcl-2 and Caspase-3 expression in BaP-induced Wistar rats (Rattus norvegicus).

Methods: This experiment used a post-test-only control group design. Female Wistar rats (n=25) were divided into a healthy control group without exposure to the carcinogenic substance B[a]P, which is used to induce cancer; a negative control group receiving only B[a]P (0.3 mg/kg BW/day); and three treatment groups receiving B[a]P plus kitolod extract at doses of 100, 200, or 300 mg/kg BW/day for 30 days. Phytochemical screening was used to characterize the extracts. Cervical histopathology and immunohistochemistry were performed to assess Bcl-2 and Caspase-3. Data were analyzed using one-way ANOVA with Tukey's HSD post hoc test ($p<0.05$).

Results: Compared with the negative control, kitolod extract significantly reduced Bcl-2 expression ($p<0.001$) and increased Caspase-3 expression ($p<0.001$), with the 300 mg/kg BW dose showing the greatest change.

Conclusion: Ethanol extract of kitolod leaves modulated Bcl-2 and Caspase-3 expression in mice exposed to B[a]P, suggesting that the extract promoted activation of the intrinsic apoptotic pathway during early cervical carcinogenesis.

Keywords: bcl-2; caspase-3, benzo[a]pyrene, cervical cancer, Hippobroma longiflora (L.) G.Don.

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INTRODUCTION

Cervical cancer is one of the major gynecological malignancies with persistently high incidence and mortality rates worldwide, particularly in developing countries.[1,2] Most of cervical cancer is caused by Human Papillomavirus (HPV) Infection.[3] Cervical cancer ranks as the second most common cancer in women after breast cancer, with the majority of new cases being diagnosed at an advanced stage.[4,5] This condition highlights the limited effectiveness of current early detection programs and the restricted availability of therapeutic options, thereby underscoring the urgent need for the discovery of novel chemopreventive and therapeutic agents that are both more effective and safer.[6,7]

Benzo[a]pyrene (B[a]P) is a well-established environmental carcinogen commonly used in experimental models to induce tumor development. Exposure to B[a]P promotes DNA damage, increases cellular proliferation, and accelerates malignant transformation in a dose-dependent manner.[8] This compound has been shown to induce DNA damage, increase gene mutations, and suppress apoptotic mechanisms, thereby facilitating the transformation of normal cells into malignant phenotypes.[9,10]

Apoptosis is a major form of programmed cell death.[11] Tumor growth occurs when there is a disruption in the balance between proliferation and apoptosis, namely when proliferation increases or apoptosis decreases, or both, or in other words, high proliferation accompanied by low apoptosis.[12] B-cell CLL/lymphoma 2 (Bcl-2) is encoded by the BCL2 gene, Bcl-2 is the founding member of the Bcl-2 family of regulator proteins which inhibit or induce apoptosis. Bcl-2 itself inhibits apoptosis.[13,14]

In other hand, Caspase-3 is a member of the family of cysteine-aspartic acid proteases, and is encoded by the CASP3 gene. Following the occurrence of apoptotic signaling, Caspase-3 playing a central role, initiates the caspase cascade specific to the apoptotic pathway.[13]

Over the past two decades, there has been increasing attention on the use of natural products as anticancer agents. One plant with notable pharmacological potential is kitolod *(Hippobroma longiflora L.G. Don)*.[15,16] Kitolod leaves contain various bioactive compounds, including flavonoids, alkaloids, saponins, and polyphenols, which have been reported to possess antioxidant, anti-inflammatory, and anticancer activities.[17,18] These compounds are believed to induce apoptosis by suppressing the expression of anti-apoptotic proteins while simultaneously enhancing the expression of pro-apoptotic proteins.[14,19] Kitolod showed that the substance had moderate potential to inhibit the growth of HeLa cells in plates with IC50 value of 227 µg/mL. [20] Therefore, investigating the effects of ethanol extract of kitolod leaves on apoptosis regulation through Bcl-2 and Caspase-3 expression in a benzo[a]pyrene-induced animal model is of great importance to explore the potential of this plant as a candidate for alternative cervical cancer therapy.

METHODS

This laboratory-based experimental study used a post-test-only control group design. The experiment was conducted in the Pharmacology Laboratory and the Anatomical Pathology Laboratory, Universitas Diponegoro, Indonesia, from June to December 2024. Ethical approval was obtained from the Health Research Ethics Committee, Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia (No. 01/EC/H/FK-UNDIP/I/1/23).

Kitolod leaves *(H. longiflora (L.) G. Don)* were collected from the Baturaden area, Central Java, Indonesia. The Kitolod used in this experiment was taxonomically verified in the Plant Laboratory, Department of Biology, Universitas Diponegoro.

To prepare the ethanol extract, the leaves were washed, oven-dried at 40–50°C, and ground into powder. 100 g of the powder was soaked in 96% ethanol (1:10 w/v) for 3–5 days. The bath was agitated daily to ensure complete extraction. The bath was then filtered. The filtrate was collected. The filtrate was concentrated under reduced pressure at 40–50°C to obtain a crude extract.

The next step was: Twenty-five healthy female Wistar rats (aged 6–8 weeks, weighing 150–200 g) were acclimatized for one week under controlled laboratory conditions. The rats were randomly divided into five groups (n=5 per group): Healthy Control (HC): no B[a]P, no extract. Negative Control (NC): only B[a]P. T1: B[a]P + extract 100 mg/kg BW/day. T2: B[a]P + extract 200 mg/kg BW/day. T3: B[a]P + extract 300 mg/kg BW/day.

B[a]P was administered orally at a dose of 0.3 mg/kg BW/day for 30 consecutive days. This dose and duration have been reported to induce cervical epithelial dysplasia and early carcinogenic changes in rodent models. B[a]P was dissolved in corn oil and administered via oral gavage to ensure accurate dosing. In the treatment group, kitolod extract was administered once daily after B[a]P administration to maintain consistent co-exposure.

At the end of the treatment period (day 31), the animals were euthanized according to standard laboratory animal ethics procedures. Cervical tissue was prepared for histopathological examination. The tissue was fixed in 10% formalin and processed into paraffin blocks. and sectioned at 3–4 µm. Hematoxylin–eosin staining was performed to assess epithelial thickness, cellular atypia, nuclear pleomorphism, and architectural disorganization.

For Immunohistochemistry (IHC) Examination, tissue sections of 4–5 µm thickness were prepared using a microtome and mounted on poly-L-lysine-coated slides. Tissue slides were subjected to deparaffinization, rehydration, and antigen retrieval using citrate buffer (pH 6.0). The sections were then incubated with primary antibodies against Bcl-2 and Caspase-3, followed by biotin-labeled secondary antibodies. Antigen visualization was performed using 3,3'-diaminobenzidine (DAB) as chromogen, and counterstaining was carried out with haematoxylin.[21] The preparations were examined under a light microscope at 400× magnification. Slides were examined by two independent blinded observers.

Bcl-2 and Caspase-3 protein expression was evaluated based on the Allred scoring system.[22] The number of cells showing positive nuclear or cytoplasmic staining and the intensity of the staining. Data are presented in figures and tables. The Allred score table was then analyzed using one-way ANOVA, followed by a Tukey-HSD post hoc test to determine significant differences

between groups. A significance level of $p < 0.05$ was considered statistically significant.

RESULTS

Cervical tissues in the healthy control group exhibited normal epithelial layering and preserved structural integrity. In contrast, the negative control group (B[a]P only) demonstrated features consistent with early cervical carcinogenesis, including epithelial thickening, nuclear atypia, and disorganized epithelial architecture.

The histopathological features of of Bcl-2 and Caspase-3 protein expression in cervical cancer tissues of female Wistar rats, were observed in both control and treatment groups. The results of IHC staining are presented in Figure 1.

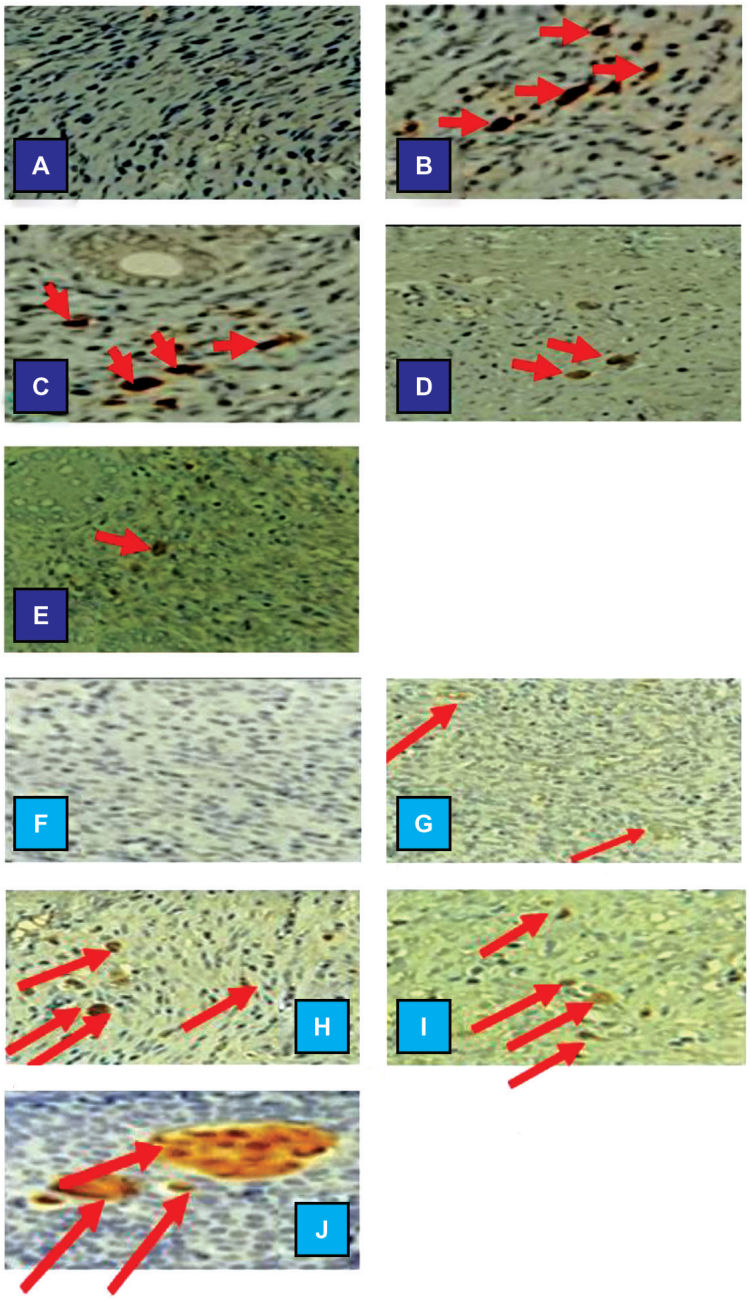

Figure 1. A to E are Bcl-2 expression in cervical cancer tissues of female Wistar rats observed at 400× magnification (red arrows indicate positive staining). A: Healthy control (HC), B: Negative control (NC), C: Treatment group 1 (T1), D: Treatment group 2 (T2), E: Treatment group 3 (T3). Figure 2. Caspase-3 expression in cervical cancer tissues of female Wistar rats observed at 400× magnification (red arrows indicate positive apoptotic staining). F to J are Caspase-3 expression in cervical cancer tissues of female Wistar rats observed at 400× magnification (red arrows indicate positive staining. F: Healthy control (HC), G: Negative control (NC), H: Treatment group 1 (T1), I: Treatment group 2 (T2), J: Treatment group 3 (T3).

Table 1. Mean Allred Scores of Bcl-2 and Caspase-3 Expression in Cervical Tissues of Wistar Rats

Group	Treatment Description	Bcl-2 (Mean ± SD)	Caspase-3 (Mean ± SD)
HC	Healthy control	2.8 ± 0.75	2.8 ± 0.75
NC	Negative control (B[a]P only)	7.2 ± 0.75	3.2 ± 0.75
T1	B[a]P + *H. longiflora* 100 mg/kg BW	6.6 ± 1.02	4.2 ± 0.75
T2	B[a]P + *H. longiflora* 200 mg/kg BW	5.0 ± 0.63	6.4 ± 1.02
T3	B[a]P + *H. longiflora* 300 mg/kg BW	3.8 ± 0.75	7.2 ± 0.75

Values are presented as mean ± standard deviation (SD). HC: healthy control (no B[a]P exposure); NC: negative control (B[a]P only). T1, T2, and T3 represent treatment groups receiving *Hippobroma longiflora* extract at doses of 100, 200, and 300 mg/kg BW/day, respectively. Bcl-2 expression represents anti-apoptotic activity, whereas Caspase-3 expression reflects pro-apoptotic activity.

DISCUSSION

Histopathological examination using H&E staining showed that cervical tissues exposed to B[a]P typically exhibited dysplasia, hyperplasia, and atypical mitotic figures, confirming that administration of B[a]P for 30 days can induce early cervical carcinogenesis. Following the administration of kitolod extract, improvements in epithelial architecture and reductions in abnormal cells were observed.

Immunohistochemical examination of cervical tissue for Bcl-2 demonstrated modulation by kitolod extract. In healthy controls, low Bcl-2 expression was observed, whereas negative controls showed a marked increase in Bcl-2 expression. Administration of kitolod extract resulted in a dose-dependent decrease in Bcl-2 expression, with the greatest reduction observed in the T3 group. In the early stages, this finding may indicate decreased anti-apoptotic activity in tissues exposed to B[a]P. These results suggest that anticancer activity through the induction of apoptosis is a key effect of bioactive compounds derived from medicinal plants.

Conversely, immunohistochemical analysis of Caspase-3 expression in Wistar rat cervical tissue revealed a progressive increase in Allred scores across the groups, in the following order: healthy control, negative control, T1, T2, and T3. The highest expression of Caspase-3 was observed in the T3 group. As Caspase-3 functions as an executioner caspase, this finding suggests enhanced regulation of apoptosis in cervical tissue following administration of increasing doses of kitolod extract. These alterations indicate activation of the intrinsic (mitochondrial) apoptotic pathway.

However, these findings are not conclusive. Previous studies have reported that clinicopathological factors and baseline Caspase-3 expression prior to neoadjuvant chemotherapy cannot predict treatment success.[23] On the other hand, the observed trend of decreased Bcl-2 expression alongside increased Caspase-3 expression is consistent with other studies reporting a significant relationship between Bcl-2 and Caspase-3 expression in immunohistochemical analyses of ovarian cancer patients.[24]

These findings should be interpreted with caution. The balance or ratio between proliferation markers and apoptosis markers may provide a more meaningful explanation of tumor growth than either parameter alone. A substantial body of literature has demonstrated the interrelationship between cell proliferation and apoptosis. These studies suggest that cancer progression in various organs is associated with dysregulation of both processes, including increased proliferation and altered apoptotic activity.[12]

Kitolod contains flavonoids, steroids, tannins, saponins, alkaloids, and polyphenols, which exhibit antioxidant and antiproliferative properties. The proposed mechanism involves reduction of oxidative stress, stabilization of mitochondrial membranes, and modulation of apoptosis-related gene expression. This may lead to decreased Bcl-2 expression and increased Caspase-3 expression, thereby directing DNA-damaged cells toward programmed cell death.

The reduction in Bcl-2 expression in the treatment groups reflects diminished survival signaling, which is important because overexpression of Bcl-2 is commonly associated with apoptosis resistance and cancer progression. Increased Caspase-3 expression indicates activation of the execution phase of apoptosis, as Caspase-3 activation leads to DNA fragmentation

and degradation of structural proteins. Recent studies have reported that natural antioxidant compounds can downregulate Bcl-2 and upregulate Caspase-3 in various cancer models.[25]

Study limitations; this study assessed only two biomarkers (Bcl-2 and Caspase-3) and used a relatively short co-exposure period; therefore, mechanistic interpretation remains limited, and long-term tumor progression was not evaluated. The sample size per group was small (n = 5), and individual variation in B[a]P metabolism may have influenced the observed biological responses. Future studies should incorporate additional mechanistic biomarkers, such as Bax/Bcl-2 ratios and oxidative stress markers, along with longer follow-up periods and standardized digital image analysis methods. Further research is needed to isolate active compounds, confirm these findings in additional experimental models, and evaluate long-term safety and optimal dosing before considering translational applications in cervical cancer prevention. Additional validation using carcinogenesis models with actual HPV exposure is also required to strengthen clinical relevance in human cervical cancer.

CONCLUSION

The main finding of this study is that *H. longiflora (L.)* G. Don leaf extract significantly reduces the anti-apoptotic marker Bcl-2 and enhances the apoptosis executor Caspase-3 in the cervical tissues of Wistar rats exposed to B[a]P, with the strongest effect observed at the 300 mg/kg dose. These results provide preliminary preclinical evidence that the extract may beneficially modulate key molecular markers involved in early carcinogenic processes. Further studies are required to identify the active compounds in kitolod associated with its anticancer properties and to evaluate long-term safety and optimal dosing before considering its application in carcinogenesis models in vivo.

ACKNOWLEDGMENTS

The authors have no funding to declare.

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