Original Article

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Cervical Cancer Chemoprevention Using *Hippobroma longiflora* Extract through Modulation of Ki-67 and p53 in Benzo[a]pyrene-Induced Wistar Rats

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Abstract

Background: Cervical cancer remains one of the most common malignancies affecting women worldwide and is closely linked to dysregulated cell proliferation and impaired tumor suppressor responses. Herbs contain effective anticancer compounds that may be used alone or as adjuvants to existing chemotherapy regimens to improve efficacy and/or reduce drug-induced toxicity. *Hippobroma longiflora* (L.) G.Don a or kitolod is a medicinal plant known to contain antioxidant and anticancer compounds. This study evaluated the effect of ethanol extract of kitolod leaves on Ki-67 and p53 expression in a Benzo[a]pyrene (B[a]P)-induced cervical carcinogenesis model in female Wistar rats.

Methods: This experimental study used a post-test-only control group design. Female Wistar rats (n=25) were grouped into five groups: a healthy control group without B[a]P exposure, a negative control group receiving only B[a]P (0.3 mg/kg BW/day); and three treatment groups receiving B[a]P combined with kitolod leaf extract at doses of 100, 200, or 300 mg/kg BW/day for 30 days. Phytochemical screening and gas chromatography–mass spectrometry (GC-MS) were used to characterize the extract. Cervical histopathologal and immunohistochemical examinations were performed to assess Ki-67 and p53 expression. Data were analyzed using one-way ANOVA followed by Bonferroni post hoc tests, with $p<0.05$ considered statistically significant.

Results: Compared with the negative control, kitolod leaf extract significantly reduced Ki-67 expression and increased p53 expression ($p<0.001$). The greatest effect was observed at the 300 mg/kg BW/day dose.

Conclusions: Kitolod leaf ethanol extract modulates Ki-67 and p53 expression in B[a]P-exposed rats, suggesting anti-proliferative activity and enhancement of tumor suppressor responses during early cervical carcinogenesis.

Keywords: Benzo[a]pyrene, cervical carcinogenesis, *Hippobroma longiflora (L.)* G.Don, Ki-67, p53

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Introduction

Cervical cancer remains a major global health problem, with an estimated 604,000 new cases and 342,000 deaths reported in 2020, making it the fourth most common cancer among women worldwide[1]. In Indonesia, cervical cancer is consistently ranked among the leading malignancies affecting women.[1,2] Although early-stage cervical cancer can be treated using surgery or radiotherapy, treatment efficacy is often limited by tumor biology, therapy-related toxicity, and restricted accessibility, particularly in low-resource settings. These limitations have encouraged growing interest in complementary therapeutic approaches that may help modulate early carcinogenic processes.[3]

Medicinal plants have been widely investigated for their potential anticancer properties because many contain bioactive compounds with antioxidant, anti-inflammatory, and anti-proliferative activities.[3,4] Such compounds may contribute to the modulation of carcinogenesis by reducing oxidative stress, attenuating proliferation, or enhancing tumor-suppressor pathways[5].

Consequently, plant-derived metabolites have been explored as protective biological agents, particularly in conditions involving chronic exposure to carcinogens that may initiate cellular dysregulation.

Hippobroma longiflora, commonly known as kitolod, is a medicinal plant from the Campanulaceae family traditionally used in several communities as a local anesthetic and for treatment of inflammation, snake bites, and tumor-related conditions.[6] Phytochemical studies have shown that kitolod leaves contain flavonoids, alkaloids, polyphenols, terpenoids, steroids, and saponins.[6,7] Flavonoids, in particular, are known for their antioxidant activity and their ability to scavenge free radicals, thereby reducing oxidative stress[8]. These biological properties support the investigation of kitolod leaf extract as a potential protective agent in carcinogenesis.

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[9]. Tumor progression is driven by an imbalance between cell proliferation and apoptosis, which can be monitored using biomarkers such as Ki-67, a marker of cellular proliferation.[10] and p53.,a tumor-suppressor protein involved in genomic stability and DNA repair.[11]

Ki-67 is widely used as a proliferation marker and predictive indicator of tumor progression. As a marker for the proliferative index, Ki-67 provides insight into the rate of tumor cell division. Additionally, Ki-67 aids in forecasting tumor's response to treatment, as faster-dividing tumors tend to respond to therapy more effectively.[12]

The p53 protein is a major tumor suppressor that functions as a transcription factor regulating metabolic pathways, a modulator of cytokine activity, and a target for cancer treatments. Due to its central role in maintaining genomic integrity during cellular stress and DNA damage, p53 is widely recognized as the "guardian of the genome".[13,14] Alterations in p53 signaling are closely associated with carcinogenesis and tumor progression.

Based on these considerations, this study aimed to evaluate the effects of ethanolic extract of *H. longiflora (L.) G. Don* leaves on cervical histopathology and Ki-67/p53 immunoexpression in Wistar rats exposed to B[a]P.

Methods

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

Leaves of Kitolod (*H. longiflora (L.) G. Don*) were collected from the Baturaden area, Central Java, Indonesia, and taxonomically verified at the Plant Laboratory, Department of Biology, Universitas Diponegoro. The leaves were washed, oven-dried at 40–50°C, and ground into powder. A total of 100 g of powdered material was macerated in 96% ethanol using a 1:10 (w/v) ratio for 3–5 days with daily agitation. The filtrate was concentrated under reduced pressure at 40–50 °C to obtain a crude ethanolic extract.[6,15] Phytochemical screening was performed using standard reagents, including Mayer, Shinoda, Froth test, and Liebermann–Burchard.

Gas chromatography–mass spectrometry (GC–MS) analysis was performed using a Shimadzu instrument with helium as the carrier gas at a flow rate of 1 mL/min. The injector and column temperatures were maintained at 260 °C and 325 °C, respectively. Compounds were identified by comparison with reference mass spectral libraries.

Twenty-five healthy female Wistar rats aged 6–8 weeks and weighing 150–200 g were acclimatized for one week under controlled laboratory conditions. Rats were randomly allocated to five groups (n=5 per group): healthy control (HC)without B[a]P exposure or extract administration; negative control (NC), receiving only B[a]P; treatment group 1 (T1), receiving B[a]P plus kitolod extract at 100 mg/kg BW/day; treatment group 2 (T2), receiving B[a]P plus kitolod extract at 200 mg/kg BW/day; and treatment group 3 (T3), receiving B[a]P plus kitolod extract at 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.[16] MAPK, topoisomerase II alpha (topoII alpha B[a]P was dissolved in corn oil and administered using oral gavage to ensure accurate dosing. In treatment groups, kitolod

extract was administered orally once daily after B[a]P exposure to maintain consistent co-exposure during the initiation and early promotion phases of carcinogenesis.

On day 31, all rats were anesthetized and humanely euthanized. Cervical tissues were excised, fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned at 3–4 µm thickness. Hematoxylin–eosin staining was performed to assess epithelial thickness, cellular atypia, nuclear pleomorphism, and architectural disorganization.

Immunohistochemical (IHC) was performed using monoclonal antibodies against Ki-67 and p53, followed by diaminobenzidine (DAB) chromogen visualization and hematoxylin counterstaining.[17] All slides were independently examined by two blinded observers.

Ki-67 and p53 expression were evaluated semi-quantitatively using the Allred scoring system.[18] The proportion score (PS) ranged from 0 to 5, where 0 indicated no positive cells (0%), 1 indicated <1%, 2 indicated 1–10%, 3 indicated 11–33%, 4 indicated 34–66%, and 5 indicated 67–100% positive cells. The intensity score (IS) ranged from 0 to 3, where 0 indicated no staining, 1 weak staining, 2 moderate staining, and 3 strong staining. Total Allred score were calculated by summing the PS and IS values, resulting in a score range of 0–8. Approximately 500 cells were evaluated per slide from at least five high-power fields (400× magnification).

Phytochemical screening results were analyzed descriptively, whereas GC–MS findings were interpreted based on chromatographic peaks analysis. Numerical data from IHC markers were tested for normality using the Shapiro–Wilk and for homogeneity of variance prior to statistical analysis. Differences among group were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni post hoc testing when appropriate. A p-value <0.05 was considered statistically significant.

Results

Phytochemical screening of the ethanolic

Table 1 Phytochemical Screening Results and GC–MS Profile of *H. longiflora (L.) G. Don* Leaf Ethanol Extract

Phytochemical Screening Results

No	Reagent	Results	Appearance
1	Mayers	+ (alkaloids)	A yellowish white precipitate forms
2	Shinoda	+ (flavonoids)	Yellow precipitate forms
3	Froth Test	+ (saponins)	Formation of foam
4	Lieberman-Burchard	+ (terpenoids)	The red color changes to blue and green.

GC–MS profile of *H. longiflora (L.) G. Don* Leaf Ethanol Extract

P	Retention Time (min)	Area (%)	Peak Area	Compound
1	30.741	3.28	22,419,456	9,12,15-Octadecatrienoic acid, methyl ester,
2	31.1	0.95	10,84 4,930	Hexadecanoic acid, methyl ester
3	33.11	34.41	122,010,474	Hexadecanoic acid, ethyl ester
4	34.042	3.7	24,236,852	9-Octadecenoic acid (Z)-, methyl ester (CAS)
5	34.95	3.69	50,202,429	11-Octadecenoic acid, methyl ester
6	36.05	0.41	3,87 9,873	Eicosanoic acid, 2-hydroxyethyl ester (CAS)
7	36.288	0.56	3,80 8,272	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)
8	37.214	1.95	24,631,149	Eicosanoic acid, methyl ester (CAS)
9	38.023	2.57	32,93 9,307	Oleic acid, 3-hydroxypropyl ester (CAS)
10	38.617	4.43	54,87 4,787	Triacontanoic acid, methyl ester

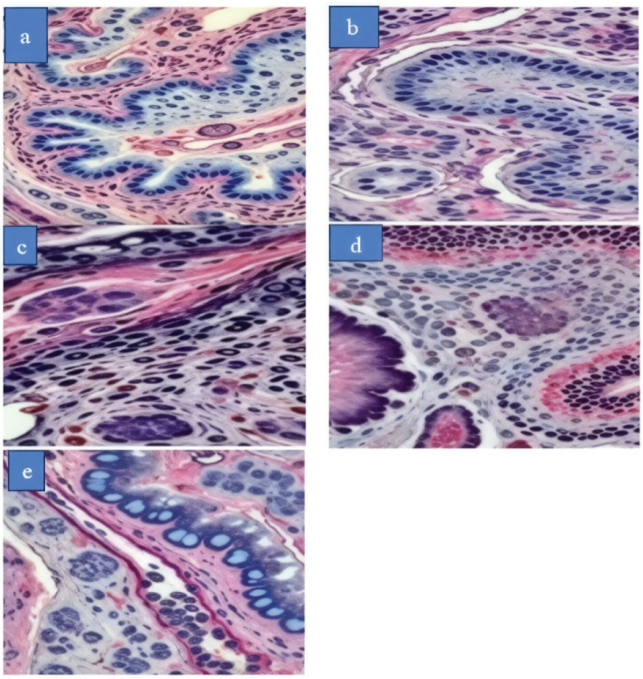

Figure 1 Representative Histopathological Images of Cervical Tissues Stained With Hematoxylin–Eosin (400× magnification)

Note: (a) healthy control (HC) showing preserved epithelial architecture; (b) negative control (NC; B[a]P only) showing epithelial thickening, cellular atypia, and architectural disorganized; (c) Treatment group 1 (T1) (B[a]P+ H. longiflora (L.) G.Don extract 100 mg/kg BW/day) showing reduced atypical changes; (d) Treatment group 2 (T2) (B[a]P+ H. longiflora (L.) G.Don extract 200 mg/kg BW/day) showing improved epithelial organization; (e) Treatment group 3 (T3) (B[a]P+ H. longiflora (L.) G.Don extract 300 mg/kg BW/day) showing near-normal epithelial morphology

extract of *H. longiflora (L.) G. Don* leaves showed the presence of alkaloids, flavonoids, terpenoids, and saponins, as indicated by characteristic colour changes in each reagent test (Table 1). GC–MS analysis identified ten major constituents, dominated by fatty acid methyl and ethyl esters as well as long-chain fatty acid derivatives, including hexadecenoic acid and octadecatrienoic acid esters.

Histopathological examination revealed preserved epithelial architecture and normal cervical morphology in the healthy control group. In contrast, the negative control group exposed to B[a]P demonstrated epithelial thickening, nuclear atypia, and disorganized epithelial arrangement, indicating early cervical carcinogenic changes. Administration of *H. longiflora (L.) G. Don* extract attenuated these pathological changes in a dose-dependent manner. The highest treatment dose (300 mg/kg BW/day) showed the most substantial improvement, with cervical morphology approaching that of the healthy control group (Figure 1).

Immunohistochemical staining revealed low Ki-67 nuclear positivity in the healthy control group, whereas the negative control group showed markedly increased nuclear staining, indicating enhanced proliferative activity following B[a]P exposure. Administration of *H. longiflora (L.) G. Don* ethanol extract progressively reduced Ki-67–positivity across treatment groups, with the lowest expression observed in the 300 mg/kg BW group (Figure 2).

Immunohistochemical staining for p53 showed low nuclear positivity in both the healthy control and negative control groups, indicating minimal baseline expression. In contrast, treatment with *H. longiflora (L.) G. Don* extract produced a dose-dependent increase in p53-positive nuclei, with the strongest staining observed in the 300 mg/kg BW/day group (Figure 3).

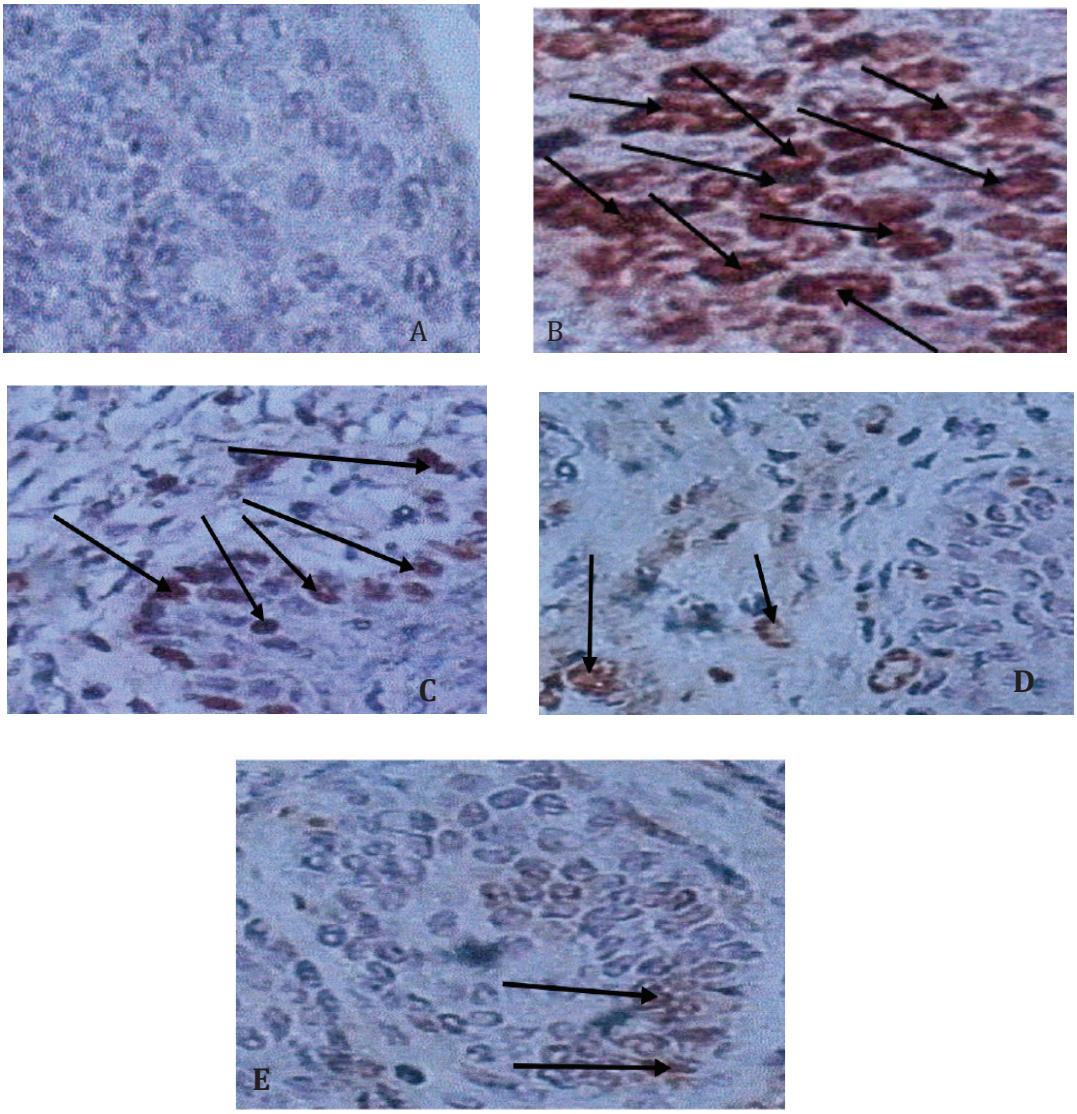

Figure 2 Ki-67 Immunohistochemical Staining of Cervical Tissues (400× magnification)

Note: (A) Healthy Control showing low Ki-67 positivity; (B) Negative Control showing strong Ki-67 nuclear staining; (C) Treatment group (T1) showing reduced Ki-67 expression compared with the negative control; (D) Treatment group (T2) showing further reduction; (E) Treatment group (T3) showing the lowest Ki-67 positivity among treatment groups

Quantitative analysis of Ki-67 and p53 immunoexpression was performed using the Allred scoring system. Mean scores and standard deviations for each group are presented in Figure 4 and 5.

The mean Ki-67 Allred score was significantly higher in the negative control (7.2±0.84) group compared with the healthy control group (2.8±0.80). Administration of *H. longiflora (L.) G. Don* extract led to a progressive reduction in Ki-67 expression across treatment groups). with mean scores of 6.0 ± 1.00 in T1, 4.0±0.71 in T2, and 3.2±0.84 in T3. The T3 group demonstrated Ki-67 levels comparable to the healthy control group ($p<0.001$).

The mean p53 Allred score in the negative control group (3.2±0.84) was slightly higher than that of the healthy control group (2.8±0.84), although the difference was not statistically significant. Treatment with *H. longiflora (L.) G. Don* extract significantly increased p53 expression in a dose-dependent manner, with mean scores of 4.2±0.84 in T1, 5.8±1.30 in T2, and 6.8±1.30 in T3. The T2 and T3 groups demonstrated significantly higher

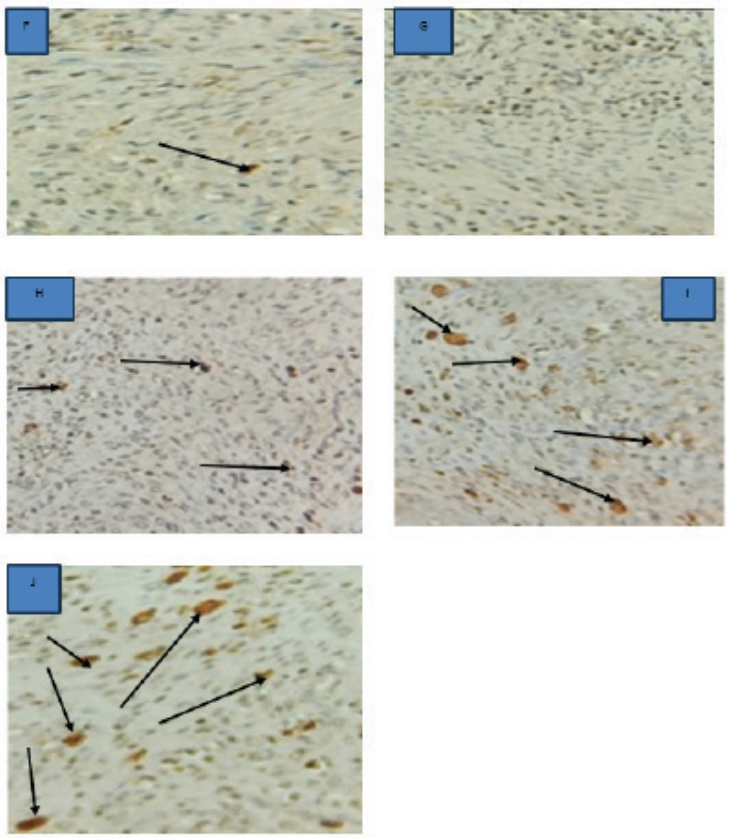

Figure 3 p53 Immunohistochemical Staining of Cervical (400×magnification)

Note: (F) Healthy Control (HC); (G) Negative Control (NC); (H) Treatment 1 (T1); (I) Treatment 2 (T2); (J) Treatment 3 (T3). Brown nuclear staining indicates p53 positivity. Treated groups show higher p53 positivity than negative control

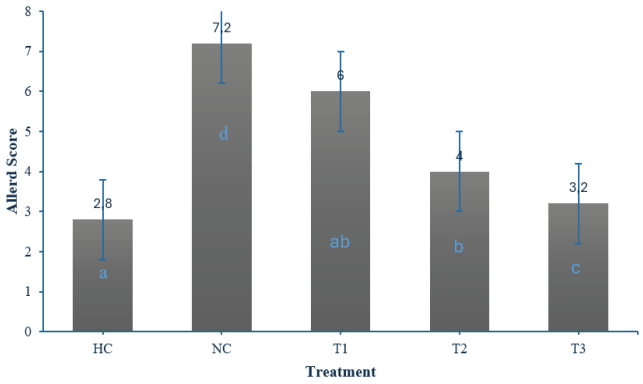

Figure 4 Mean Allred Scores of Ki-67 Expressions in Cervical Tissues of Wistar Rats

Note: Bars represent mean ± SD. Different letters inside bars indicate statistically significant differences between groups based on one-way ANOVA ($p<0.001$) followed by Bonferroni post hoc analysis ($p < 0.05$). HC: Healthy Control (2.8±0.80); NC: Negative Control (7.2±0.84); T1 (6.0±1.00); T2 (4.0±0.71); T3: (3.2 ±0.84): treatment groups receiving H. longiflora (L.) G. Don extract at 100, 200, and 300 mg/kgBW

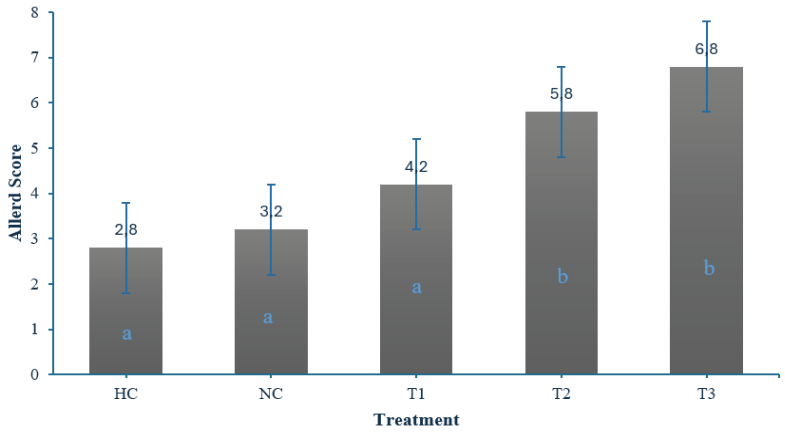

Figure 5 Mean Allred Scores of p53 Expression In Cervical Tissues of Wistar Rats

Note: Bars represent mean ± SD. Different letters inside bars indicate statistically significant differences between groups based on one-way ANOVA ($p < 0.001$) followed by Bonferroni post hoc analysis ($p < 0.05$). HC: Healthy Control (2.8±0.84); NC: Negative Control (3.2±0.84); T1 (4.2±0.84); T2(5.8±1.30); T3 (6.8±1.30): treatment groups receiving H. longiflora (L.) G. Don extract at 100, 200, and 300 mg/kg BW

p53 expression compared with both control groups ($p<0.001$).

Discussion

Phytochemical screening demonstrated that the ethanolic extract of *H. longiflora* leaves contained alkaloids, flavonoids, terpenoids, and saponins. These findings indicate the presence of several classes of secondary metabolites commonly associated with cytotoxic, antioxidant, and antiproliferative effects. [6,7,19]

In contrast, GC–MS analysis predominantly identified volatile and semi-volatile compounds, mainly fatty acid methyl esters, ethyl esters and long-chain fatty acid derivatives such as hexadecanoic acid and octadecatrienoic acid esters. This difference reflects the analytical specificity of GC-MS, which primarily detects volatile constituents. Many flavonoids and phenolic compounds are non-volatile and generally require prior derivatization before GC–MS analysis. Therefore, the absence of flavonoids in the chromatographic profile does not contradict the phytochemical screening results. Instead, both analyses should be considered complementary, with phytochemical screening capturing broader classes of non-volatile metabolites and GC–MS characterizing the volatile fraction of the extract.

Immunohistochemical analysis revealed that the negative control group exhibited significantly increased Ki-67 expression than the healthy control group, indicating increased proliferative activity following carcinogen exposure. Treatment with *H. longiflora (L.) G. Don* extract progressively reduced Ki-67 expression across treatment groups, with the lowest Allred score observed in the 300 mg/kg BW/day group. Ki-67 is a well-establisehd marker of cellular proliferation that is expressed all active phases of the cell cycle, but absent in resting cells[20]. Ki-67 is considered a meaningful biomarker for tumor progression and potential therapeutic targeting. [11,21]Prathima institute of medical sciences in South India, between August 2018 to July 2020.\nResults: The mean age of diagnosis for benign, borderline and malignant epithelial tumors was 42years, 49 years, 56years respectively. Most common histological type was serous epithelial tumors (50%.

Interpretation of p53 immunohistochemical findings should be approached cautiously. Under normal physiological conditions, p53 is present at low levels; however, in response to DNA damage or cellular stress, p53 accumulates in the cell nucleus and carries out pro-apoptotic functions [22]. In this study, administration of *H. longiflora (L.) G. Don* extract resulted in a dose-dependent increase in p53 expression. The T2 and T3 groups showed significantly higher p53 Allred scores compared with both the healthy and negative control groups, indicating enhanced activation of tumor-suppressor pathways at higher

extract doses.

Nevertheless, increased p53 expression should not be interpreted as direct evidence of apoptosis because p53 activation may also reflect DNA-damage responses, cell-cycle regulation, or non-apoptotic cellular stress pathway.[23] Moreover, IHS detection of p53 does not necessarily linked to TP53 mutation status. Another study has reported that certain TP53 nonsense mutations may produce absent or weak staining due to degradation of abnormal transcripts[24]and the presence of TP53 mutation discriminates between high and low-grade serous carcinomas and is now an important biomarker for clinical trials targeting mutant p53. p53 immunohistochemistry (IHC. Consequently, additional apoptosis-specific assessments are required to clarify the biological significance of increased p53 expression in this model.

The observed biological effects may be related to the phytochemical constituents detected in the extract. Flavonoids, one of the major polyphenolic groups identified, possess strong antioxidant properties and are known to scavenge free radicals, thereby protecting cells from oxidative stress-induced damage.[7,15] In addition, flavonoids, alkaloids, and phenolic compounds have been widely reported to exhibit cytotoxic and antiproliferative effects against various cancer cells types. These mechanisms may contribute to the modulation of Ki-67 and p35 expression. However, experimental evidence specifically investigating *H. longiflora (L.) G. Don* remains scarce, and the present findings provide a novel contribution to understanding its potential molecular effects in a B[a]P-induced carcinogenesis model.

Several limitations should be acknowledged**.** First, the study evaluated only two biomarkers, Ki-67 and p53, limiting mechanistic interpretation of the observed effects. Second, the realtively short exposure period did allow evaluation of long-term tumor progression. Third, GC–MS analysis may not fully represent non-volatile flavonoids and phenolic compounds without derivatization. In addition, the sample size per group was relatively small (n=5), and interindividual variation in B[a]P metabolism may have influenced the biological responses observed. Future studies should incorporate additional mechanistic biomarkers, including caspase-3, Bax/Bcl-2 ratios, and oxidative stress markers, along with longer observation periods and standardized digital image-analysis methods.

In conclusion, *H. longiflora (L.) G. Don* leaf extract significantly reduces proliferative activity (Ki-67) and enhances tumor-suppressor response (p53) in cervical tissues exposed to B[a]P, with the strongest effects observed at the 300 mg/kg BW/day dose. These findings suggest that the extract can beneficially modulate key molecular pathways involved in early cervical carcinogenesis by suppressing proliferative activity and enhancing tumor-suppressor responses. Further studies are required to isolate active compounds, confirm these findings in additional experimental models, and evaluate long-term safety and optimal dosing before potential translational applications in cervical cancer prevention.

Authors' Contributions

ABS contributed to experimental design, data interpretation, and manuscript preparation. ES and DJW contributed to data interpretation and critical manuscript revision. All authors approved the final version of the manuscript.

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Generative Ai Disclosure Statement

No generative artificial intelligence (AI) or AI-assisted technologies were used in the preparation of this manuscript.

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