RESEARCH ARTICLE

Anti-proliferative and Apoptotic Activities of Kasturi Tobacco (*Nicotiana tabacum* L.) Leaf Resinoid on Cervical Cancer Cell

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Abstract

BACKGROUND: Cervical cancer has a high rate of morbidity and mortality in women with cancer. Recent studies have found that tobacco (*Nicotiana tabacum* L.) is a potential source of anti-cancer agents. Hence, this study was conducted to determine the potential of Kasturi tobacco leaf resinoids as apoptotic agents against cervical cell malignancies, since it has not been fully elucidated before.

METHODS: The phytochemical diversity of Kasturi tobacco resinoids was generated by gas chromatography-mass spectrometry (GC-MS) analysis followed by spectral similarity to National Institute of Standards and Technology (NIST) database. Cytotoxicity and proliferative activity of HeLa cells treated with Kasturi tobacco resinoids at various concentrations were evaluated by MTT assay. The expression of Caspase-3, cyclooxygenase-2 (COX-2) and heat shock protein 90 (HSP-90) in HeLa cells was analyzed by immunocytochemistry. Next, the migration ability of HeLa cells was observed by the scratch method.

RESULTS: Kasturi tobacco resin contains 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trim with α-2,7,11-cembratriene-4,6-diol (α-CBD) structure in the form of a diterpenoid compound with the chemical formula $C_{20}H_{34}O_2$ and a molecular weight of 306 Da. Kasturi tobacco resinoid with IC_{50} value of 2500 µg/mL inhibited proliferative activity during 72 hours. At a concentration of 1¼ IC_{50} and incubation for 48 hours, Caspase-3 expression increased by 74.1%, while COX-2 and HSP-90 expression decreased by 28.3% and 26.1%, respectively. HeLa cell migration was inhibited by Kasturi tobacco resinoid at 24 hours incubation.

CONCLUSION: Kasturi tobacco resinoids with a concentration of 1¼ IC_{50} have potential as cervical anti-cancer agents by increasing Caspase-3 expression and decreasing COX-2 and HSP-90 expression within 48 hours.

KEYWORDS: Kasturi tobacco resinoids, cervical cancer, anti-cancer agent, proliferative activity

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Introduction

Kasturi tobacco (*Nicotiana tabacum* L.) is one of the tobacco varieties cultivated in Indonesia, which usually grows in dry season. Apart from its popularity in the cigarette industry, tobacco leaves is also known to have chemical compounds with biological and pharmacological activities that can be used for medical purposes.(1-4) Ethnopharmacological reports indicate that tobacco leaves have been used as traditional medicine, and recent studies have found that tobacco leaves have anti-cancer properties.(5-8)

The contribution of the bioactive components in tobacco as anti-tumor in modern medicine can not be ignored. Recently, the use of α-2,7,11-cyprotermine-4,6-diol (α-CBD) from tobacco has been evaluated in hepatocellular carcinoma cells *in vitro*. This study shows that α-CBD decreases viability, inhibits proliferation,

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changes plasma membrane permeability, promotes apoptotic morphology, stops the cell cycle in S-phase, as well as induces the apoptosis of hepatocarcinoma cells.(6) In addition to α-CBD, flavonoids in Kasturi tobacco leaves is reported to have more cytotoxic effects on breast cancer cells than Vero cells. These results indicate that flavonoids are more selective against breast cancer cells than normal cells, so that flavonoids in tobacco leaves can be considered as potential chemotherapeutic or chemopreventive agents based on their ability to induce apoptosis in breast cancer cells with relatively low toxicity to normal cells.(7)

Cervical cancer is the fourth most common cancer in women caused by Human Papilloma Virus (HPV), which can affect cell proliferation and apoptosis.(9) Molecular studies revealed viral oncoproteins, E6 and E7, interfere with the tumor suppressor genes p53 and pRb. Subsequently, inactivation of host proteins disrupts DNA repair and apoptosis mechanisms, and forces cells to remain in a proliferative state and exit the cell cycle.(10) Cancer treatment methods vary widely, in this regard including increasing the specificity of cytotoxic agents remains a challenging developmental task. Currently, chemotherapeutic agents are administered without unsatisfactory side effects for patients. Therefore, discovery of new anti-cancer drugs that are effective, efficient and safe is in great demand, including new anticancer drugs from natural resources. It is known that natural bioactive compounds found in plants have many biological activities and anticancer effects.(11) Therefore, this study was conducted to determine the potential of Kasturi tobacco leaf resinoids as apoptotic agents against cervical cell malignancies, since it has not been fully elucidated.

Methods

Preparation of Tobacco Resinoid

Kasturi tobacco leaves for this study were obtained from Ledokombo village, Jember district, Indonesia. One-thousand g of Kasturi tobacco leaves were air-dried, and then grounded into tobacco leaf powder. Fifty g of tobacco leaf powder was immersed in 500 mL methanol (Merck, Darmstadt, Germany), stirred for 24 h, and then filtered. The 250 mL n-hexane (Merck) was added to the filtrate and then separated and treated with 0.5 mL of 10% HCl solution (Merck). After the acid layer was removed, the hexane fraction was tested with Dragendorff's reagent (ACS Chemicals, Gujarat, India) to determine the alkaloid content. Finally, the alkaloid-free hexane fraction was evaporated with a vacuum rotary evaporator.(12) Then, 1 g of tobacco resinoids was fractionated using a silica column chromatography (2.5x30 cm) containing silica gel (Sigma-Aldrich, Burlington, MA, USA) with five solvents. Furthermore, the fractions were subjected to gas chromatography-mass spectrometry (GC-MS) analysis.(12)

GC-MS Analysis

Ten mg of tobacco resinoid was dissolved in 10 mL dichloromethane (Merck) to produce a sample solution. One µL sample was injected into GC-MS for 2 min at 160°C, then the temperature was increased at 10°C to 210°C/min and maintained for 35 min. This step was repeated with an increase in temperature of 10°C to 250°C/min and maintained for 15 min. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. Data were analyzed using GC-MS software equipped with National Institute of Standards and Technology (NIST) 08 and Wiley7 libraries (Shimadzu LabSolutions, Kyoto, Japan).(12)

Cell Culture

Human cervical cancer cells (HeLa cells) was obtained from Laboratory of Molecular Medicine, Center for Development of Advanced Science and Technology, Universitas Jember, Indonesia. The cells were cultured and maintained in Roswell Park Memorial Institute (RPMI) (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), and 2.5 µg/mL amphotericin B (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C.

MTT Cytotoxic Assay

HeLa cells with a density of $5x10^3$ cells/well were transfered into 96-wells plate and incubated for 24 h at 37°C and 5% CO_2. Cells were treated wtih Kasturi tobacco resinoids in the concentration of 125, 250, 500, 1000, 2000, 4000, 8000, and 12000 µg/mL, and incubated for 24 h. Thereafter, 15 µL MTT reagent (BioAssay System, Hayward, CA, USA) was added to each well and incubated for four h at 37°C. Viable cells react with MTT and form purple formazan crystals. Stopper solution (BioAssay System) was added to dissolve formazan crystals and incubated for 1 h at room temperature. The plate was shaken prior to absorbance reading in a microplate reader at 570 nm.

Cell Proliferation Assay

HeLa cells with a density of $5x10^3$ cells/well were transfered into 96-wells plate and incubated for 24 h at 37°C and 5% CO_2. The cells were treated with Kasturi tobacco resinoids

with a serial concentration of ¼ inhibitory concentration $(IC)_{50}$, ½ IC_{50}, ¾ IC_{50}, 1 IC_{50}, 1¼ IC_{50} , 1½ IC_{50}, 1¾ IC_{50}, and 2 IC_{50}, then incubated for 0, 24 48, and 72 h. At the end of the incubation period, 15 µL MTT reagent was added, and incubated for 4 h at 37°C. After incubation, the MTT formazan precipitate was dissolved with a stopper solutionand shaken before reading the absorbance at 570 nm using a microplate reader.

Immunocytochemistry Analysis

HeLa cells with a density of $5x10^4$ cells/well were grown on poly-L-lysine slides in 24-well plates and incubated for 24 h at 37°C and 5% CO_2. Cells were treated Kasturi tobacco resinoids with a serial concentration ¾ IC_{50}, 1 IC_{50}, 1¼ IC_{50} and incubated for 24 and 48 h. Cells were washed with phosphate buffered saline (PBS) and fixed with cold methanol at 4°C for 10 min. Cells were then washed with PBS, quenched by adding 100 µL hydrogen peroxide blocking solution (ScyTek, Logan, UT, USA) and incubated for 10 min at room temperature. Each well was added 100 µL Caspase-3 (Abbiotec, Escondido, CA, USA), cyclooxygenase-2 (COX-2) (Santa Cruz Biotechnology, Dallas, TX, USA), or heat shock protein 90 (HSP-90) (Novusbio, Centennial, CO, USA) primary antibodies and incubated overnight at 4°C. Cells were washed four times with PBS, added 100 µL secondary antibodies (Scytek), and incubated for 15 min at room temperature. Cells were washed four times with PBS, added with 100 L Streptavidin/HRP (Scytek) and incubated for 20 min at room temperature, and washed. Chromogenic solution of 100 µL 3,3-diaminobenzidin (DAB) (Scytek) was added to each well, and incubated for 10 minutes. Cells were stained with haematoxylin (Sigma-Aldrich) for three minutes, and washed. Protein expression was characterized by brown color in the cells observed with a light microscope model Olympus CX31RBSFA (Olympus, Tokyo, Japan) and Optilab camera (Optilab, Phoenix, AZ, USA). Data were analyzed using the Immunoratio feature in the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The data obtained were in the form of percentage values of antibodies expressed on cells.

Migration Assay

HeLa cells with a density of $1x10^5$ cells/well were seeded in 24-wells plate and incubated 24 h at 37°C and 5% CO_2. Monolayer cells were scratched with a sterile pipette of 200 µL. Every well was washed PBS to remove the loose cells when scratching. Cells were treated with 200 µL Kasturi tobacco resinoids with a concentration of ¾ IC_{50}, 1 IC_{50}, 1¼ IC_{50}. Cell migration was observed under a light microscope with a magnification of 40x at 0, 24, and 48 h.

Statistical Analysis

Data were expressed as a mean±standard deviation (SD) with Microsoft Excel ver. 16.14.1 (Microsoft Corporation, Redmond, WA, USA). Statistical significance was analyzed with one-way ANOVA, followed by Tukey's post-hoc multiple-comparison test of significance between different group by SPSS ver. 22.0 (IBM Corporation, Armonk, NY, USA). The $p<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of Chemicals Composition by GC-MS Analysis

The GC-MS chromatogram showed 50 distinct peaks represent various individual chemical components at 60 min of running time (Figure 1). The chemical composition of Kasturi tobacco resinoids were characterized by chemical compound class and presented as a percentage of the total compound. Kasturi tobacco resinoids were composed of esters (22%), hydrocarbons (20%), terpenoids (12%),

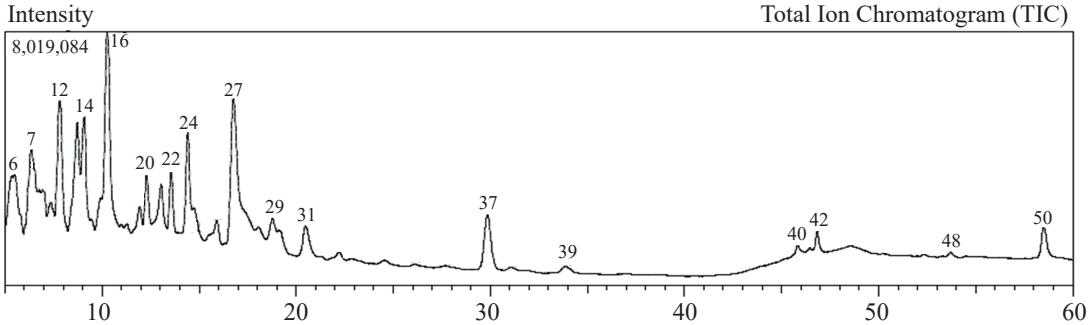

Figure 1. Chromatogram of Kasturi tobacco resinoids determined by GC-MS.

alcohols (10%), phenols (6%), haloalkanes (4%), ethers (4%), and other minor compounds (22%). Bioactive compound targets were found at the 25th and 26th peaks at 14,742 and 15,918 retention times. This compound was known as 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trim with α-CBD structure.

Cytotoxic and Proliferation Activity of Kasturi Tobacco Resinoid on HeLa Cells

Kasturi tobacco resinoids had cytotoxic activity against HeLa cells at a concentration of 4000, 8000, and 12000 µg/mL, which could reduce HeLa cell viability by 44.94±5.95%, 23.17±0.58%, 2.98±0.69%, respectively. The IC_{50} value of 2500 µg/mL is obtained from the calculation of the percentage of non-viable HeLa cells after incubating 24 h with the addition of Kasturi tobacco resinoids (Figure 2). Cytotoxic test results showed statistically significant differences (p<0.05) at each concentration.

For analysis of proliferation activity, HeLa cells were treated by Kasturi tobacco resinoids with a concentration of 1¼ IC_{50}, 1½ IC_{50}, 1¾ IC_{50}, and 2 IC_{50}, and significant decreases were found in the cell viability at 0, 24, 48, and 72 h (Figure 3).

Expression of Caspase-3, COX-2, and HSP-90 on HeLa cells

Caspase-3, COX-2, and HSP-90 expression in HeLa cells were evaluated by immunocytochemical analysis. Kasturi tobacco resinoids increased Caspase-3 expression (p<0.05), but decreased COX-2 and HSP-90 expression (p<0.05). At 24 h incubation, HeLa cells treated with Kasturi tobacco resinoid with concentrations of ¾ IC_{50}, 1 IC_{50}, and 1¼ IC_{50} expressed Caspase-3 at 33.1%, 39.9%, and 48.6%, respectively. Caspase-3 expression became 59.3%, 66.1%, and 74.1% at 48 h. Meanwhile COX-2 was expressed by 47.2%, 41.2% and 36.2% at 24 h, and decreased to 29.3%, 29.2%, and 28.3% at 48 h. HSP-90 was expressed by 47.3%, 37.9%, and 34.3% at 24 h, and decreased to 31.3%, 29.1%, and 26.1% at 48 h (Figure 4-7).

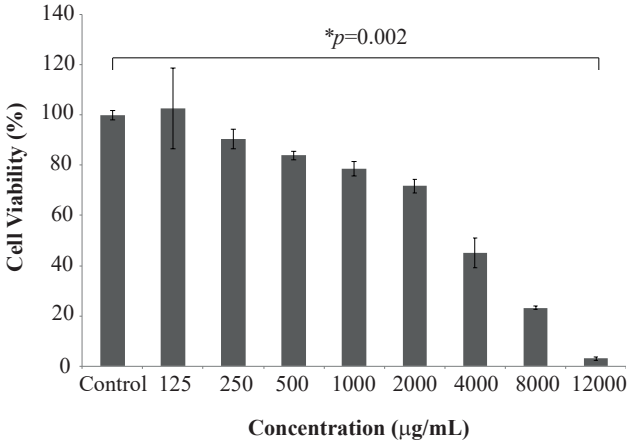

Figure 2. Viability of HeLa cells treated Kasturi tobacco resinoids with serial concentrations. Statistical analysis showed significant difference between groups. Test was repeated three times (*significant if p<0.05).

Effect of Kasturi Tobacco Resinoid on HeLa Cells Migration

HeLa cell migration was observed by comparing the filling of the scratch area for 24 and 48 h. Microscopic observations showed that HeLa cells slightly filled the scratch area within 24 h. However, HeLa cells were found to be non-viable at

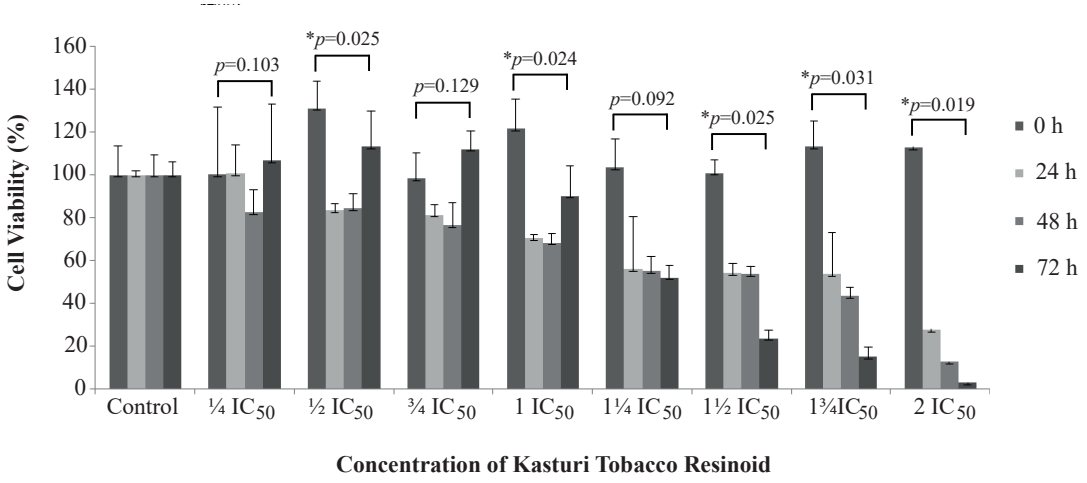

Figure 3. Proliferation of HeLa cells treated Kasturi tobacco resinoids with concentrations of ¼ IC_{50}, ½ IC_{50}, ¾ IC_{50}, 1 IC_{50}, 1¼ IC_{50}, 12/4 IC_{50}, 1¾ IC_{50}, dan 2 IC_{50} for 0, 24, 48, and 72 h. Statistical analysis showed significant difference between groups during incubation time (*significant if p<0.05).

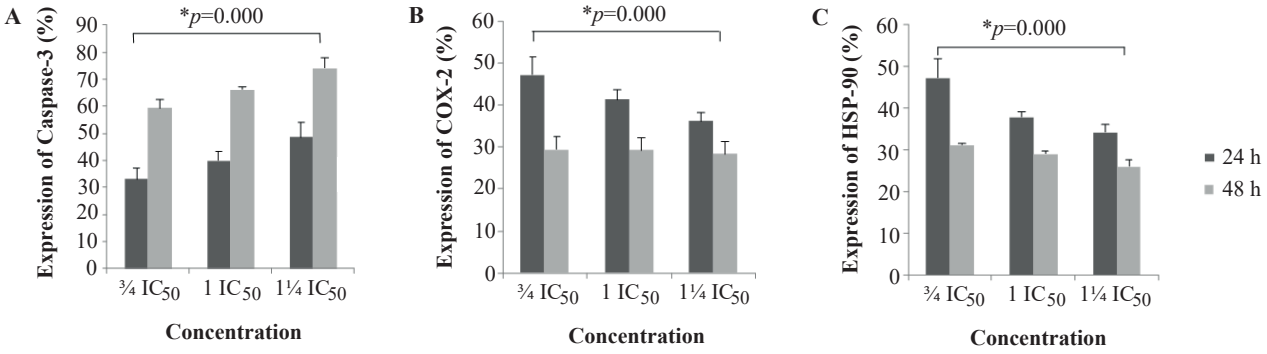

Figure 4. Effect of Kasturi tobacco resinoids on the expression of Caspase-3, COX-2, and HSP-90 in HeLa cells. A: Caspase-3 expression; B: COX-2 expression; C: HSP-90 expression. Statistical analysis showed significant difference between groups during incubation time (*significant if $p<0.05$).

48 h incubation. In contrast, HeLa cells that were not treated with Kasturi tobacco resinoid were able to completely fill the scratch area at 24 to 48 h (Figure 8).

Discussion

Chemotherapy drugs have become the main choice in the treatment of cervical cancer. The therapeutic efficacy of most anti-cancer agents in clinical use is limited by their general toxicity to proliferating cells, including some normal cells. (13) The main anti-cancer agents are still naturally sourced with varying degrees of success worldwide.(11) Tobacco leaf has several beneficial secondary metabolites which are being developed for medicinal use and reported to have potential as anti-cancer agents.(8)

This study found that Kasturi tobacco resinoids are composed of esters, hydrocarbons, terpenoids, alcohols, phenols, haloalkanes, ethers, and other minor chemical compounds. In addition, diterpene components in Kasturi tobacco resinoids analyzed by GC-MS showed that the chemical structure of cembranoid as α-CBD 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trim, which is a diterpenoid compound with the chemical formula $C_{20}H_{34}O_2$ and a molecular weight of 306 Da.(14) Cembranoids can induce cell cycle arrest in the G0/G1 and S phases, which affect cell proliferation and apoptosis in cancer cells. (15) Cembranoids from tobacco leaves infected with *Rhodococcus fascians* are known to inhibit the proliferation of human glioma cells. Tobacco leaf cembranoid can affect proliferation, change microtubule stability, and slow down cell division.(16) In addition, tobacco leaf cembranoid can inhibit the proliferation of HepG2, MCF-7, and PC-3 cells.(8,17)

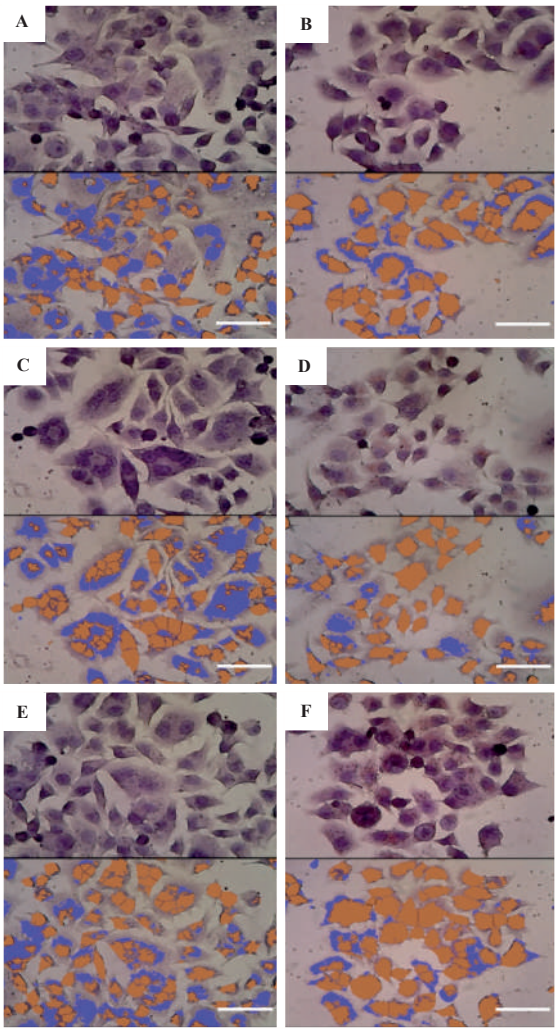

Figure 5. Expression of Caspase-3 in HeLa cells treated Kasturi tobacco resinoids during incubation of 24 and 48 h with different concentrations. A-B: ¾ IC_{50}; C-D: 1 IC_{50}; E-F: 1¼ IC_{50}. The upper box shows microscopic observations in DAB and hematoxylin-stained HeLa cells, while the lower box is an immuno-ratio analysis showing the percentage of Caspase-3 expression based on brown color in HeLa cells. White bar: 10 µm.

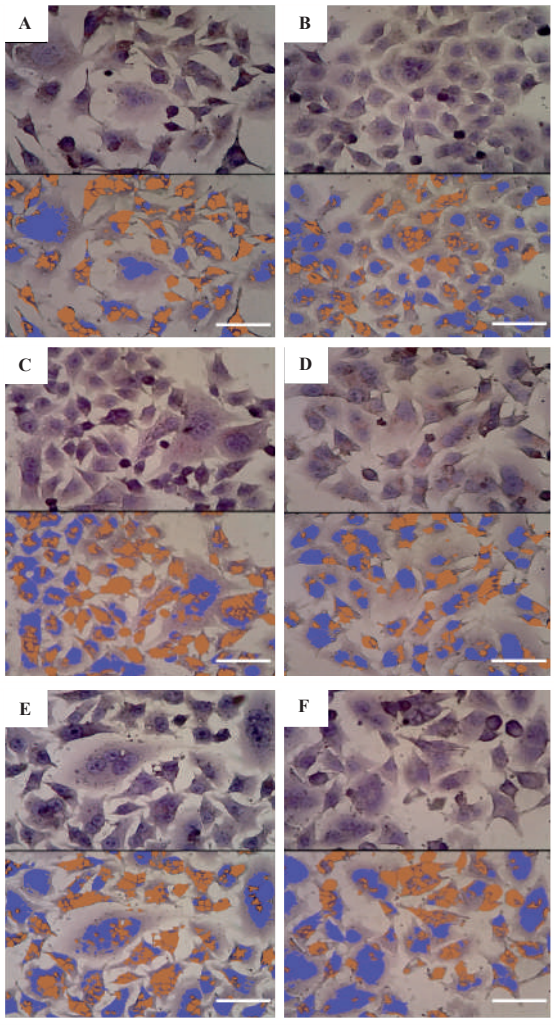

Figure 6. Expression of COX-2 in HeLa cells treated Kasturi tobacco resinoids during incubation of 24 and 48 h with different concentrations. A-B: ¾ IC_{50}; C-D: 1 IC_{50}; E-F: 1¼ IC_{50}. The upper box shows microscopic observations in DAB and hematoxylin-stained HeLa cells, while the lower box is an immunoratio analysis showing the percentage of COX-2 expression based on brown color in HeLa cells. White bar: 10 µm.

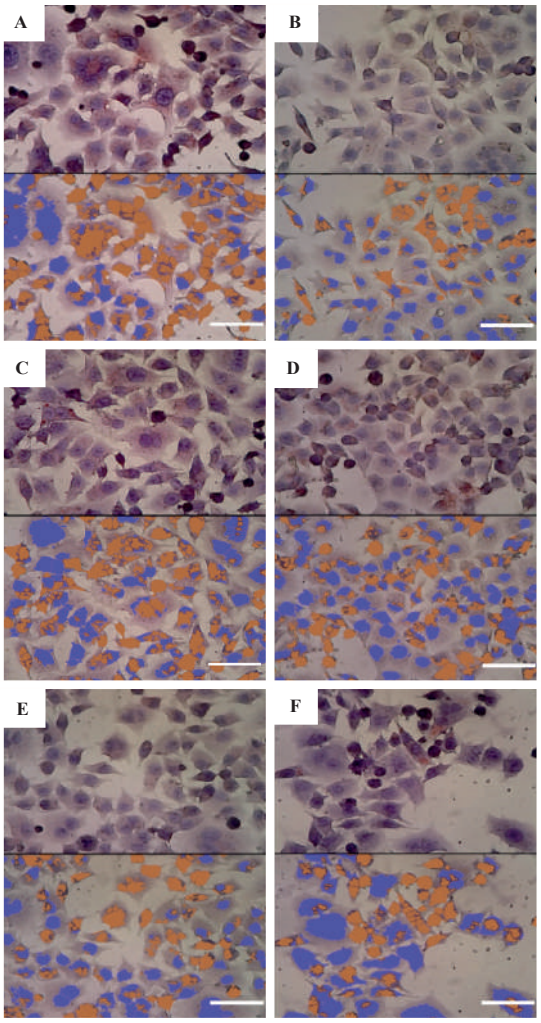

Figure 7. Expression of HSP-90 in HeLa cells treated Kasturi tobacco resinoids during incubation of 24 and 48 h with different concentrations. A-B: ¾ IC_{50}; C-D: 1 IC_{50}; E-F: 1¼ IC_{50}. The upper box shows microscopic observations in DAB and hematoxylin-stained HeLa cells, while the lower box is an immunoratio analysis showing the percentage of HSP-90 expression based on brown color in HeLa cells. White bar: 10 µm.

Unfortunately, the chromatography protocol in this study could not produce cembranoid compounds from Kasturi tobacco resinoids. This study revealed that Kasturi tobacco resinoid had a less significant cytotoxic effect on HeLa cells at IC_{50} 2500 µg/mL. This is due to the presence of other chemical compounds in Kasturi tobacco resinoids, which have been identified as having no anticancer activity. Based on IC_{50} values, Kasturi tobacco resinoids were tested for anti-proliferative activity against HeLa cells at concentrations of 1¼ IC_{50}, 1½ IC_{50}, 1¾ IC_{50}, and 2 IC_{50}. During incubation, Kasturi tobacco resinoids showed a significant decrease in HeLa cell viability from 24 to 72 h (Figure 3).

Apoptosis is a physiological process that results in cellular self-destruction. Dysregulation of apoptosis in cells plays an important role in the development of malignancies. (18) To initiate apoptosis, it is necessary to activate Caspase-3 (19,20), which plays a role in the destruction of many functional and structural proteins in cells (21,22). In this regard, therapeutic targeting of apoptosis is considered as an important strategy for developing anti-tumor agents, because cancer has the ability of cell proliferation and inhibition of apoptosis. Our research on novel and safe anti-cancer agents from natural products will elucidate the anti-cancer ability of Kasturi tobacco resinoids to induce apoptosis in HeLa cells.

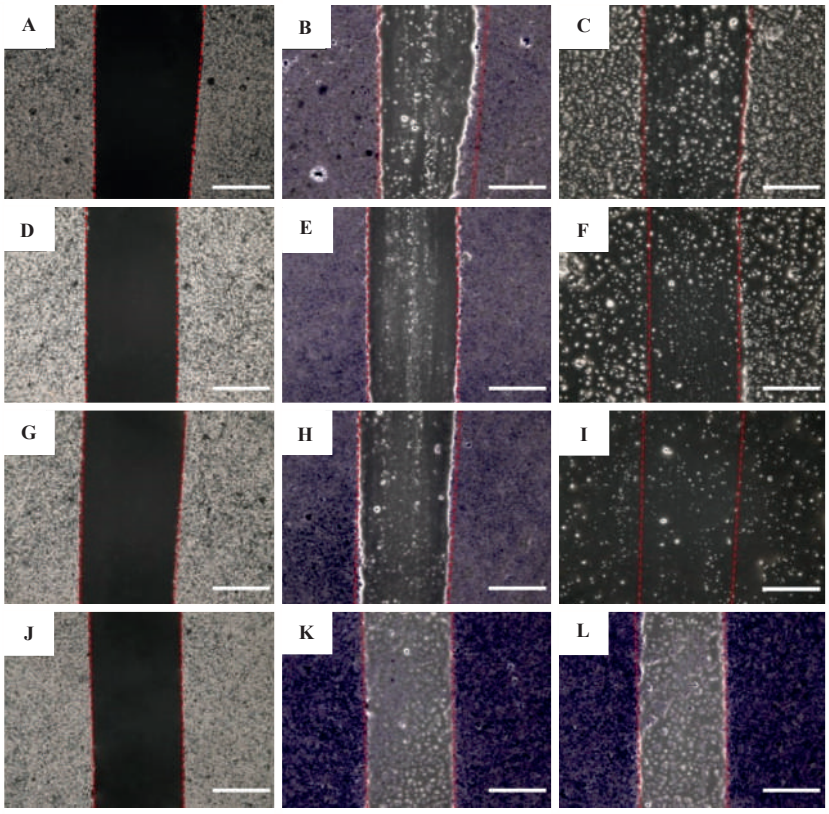

Figure 8. Cell migration activity in HeLa cells treated Kasturi tobacco resinoids during incubation of 0, 24 and 48 h with different concentrations. A-C: ¾ IC_{50}; D-F: 1 IC_{50}; G-I: 1¼ IC_{50}; J-L: Non-treated HeLa cells. HeLa cells slightly filled the scratch area within 24 h, but non-viable at 48 h incubation. In contrast, non-treated HeLa cells completely filled the scratch area at 24 to 48 h. White bar: 10 µm.

In the present study, Caspase-3 showed increased expression in a dose-dependent manner. This finding is in line with previous studies that reported that cembranoids not only suppress migration ability, but also induce apoptosis in cervical cancer cells.(15) Cembranoids induce apoptosis through mitochondrial activation by inhibiting the B-cell lymphoma 2 (Bcl-2) gene and by increasing the expression of the Bax gene.(23) Activation of this intrinsic apoptotic pathway is important in the mechanism of apoptosis induced by cytotoxic agents and oxidative stress. This mitochondrial pathway is associated with members of the Bcl family, leading to the release of Cytochrome *c* and binding to Procaspase-9 and Apoptotic protease activating factor 1 (APAF-1) which play a role in apoptosome formation and activation of Caspase-9, activation of Procaspase-3 for Caspase-3, then triggering apoptosis.(24)

The pathological condition in most cancers is exacerbated by the inflammatory process. COX-2 is an isoform of cyclooxygenase that can be induced by growth factors and cytokines, so that it is overexpressed in inflammatory conditions. The main product of the COX-2 enzyme, the prostanoids, is released from cells and acts locally in autocrine and paracrine modes, activating diverse intracellular pathways, thereby inducing cell proliferation, anti-apoptotic activity, angiogenesis, and increased metastasis. COX-2 is detected in most cervical cancers and is involved in their development, metastasis, and prognosis.(25) This study detected a decrease in COX-2 expression in HeLa cells treated with Kasturi tobacco resinoid during the incubation period of 24 and 48 h. Inhibition of COX-2 expression can increase its therapeutic potential in cervical cancer therapy.(26-28)

This study also demonstrated the effectiveness of Kasturi tobacco resinoid in inhibiting cell proliferation by reducing HSP-90 expression in HeLa cells. HSP-90 is an important chaperone protein involved in the regulation of biological processes such as cell signaling, proliferation, and survival of normal cells and cancer cells. Cells respond to environmental stress by increasing the synthesis of HSP-90.(29) Extreme environmental factors, such as hypoxia, oxidative stress, and high temperature lead to HSP production.(30,31) HSP-90 is often overexpressed in cells to maintain cancer cell viability by protecting oncoproteins that are multi-mutated and/or over-expressed, by incorrect cleavage and degradation.(32) The inhibition of HSP-90 also induces apoptosis and inhibits cervical cancer cell growth.(33)

Cell migration plays a role in cellular physiological and pathological processes including tumor metastasis initiated by cell proliferation, adhesion, migration, and

angiogenesis.(34) The migration activity of HeLa cells was analyzed by scraping the monolayer cells to form a scratch area. Cell adhesion molecules on the cell surface hold the cell from being misdirected. Adhesion molecules hold cells to other cells, cells to the extracellular matrix (ECM), and ECM to the cytoskeleton. Cell movement is triggered by chemical or physical signals detected by proteins present on the cell membrane. In this study, Kasturi tobacco resinoids were not only able to inhibit proliferation but also inhibit HeLa cell migration, compared to untreated HeLa cells. This evidence is in line with other studies revealing the ability of cembranoid to inhibit migration of human prostate cancer cells and breast cancer cells.(17) More specific extraction and fractionation are needed to obtain cembranoid compounds as cervical anti-cancer agents, and it is necessary to carry out further flowcytometry analysis to determine more accurate cell apoptotic activity.

Conclusion

Kasturi tobacco leaves contain a diterpene compound with a chemical structure of α-CBD which has been identified as 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trim, and posses anti-cancer activity. Kasturi tobacco resinoids can reduce 50% viability of cervical cancer cells with an IC_{50} value of 2500 µg/mL. Kasturi tobacco resinoids with a concentration of 1¼ IC_{50} (3125 µg/mL) have potential as cervical anti-cancer agents by increasing Caspase-3 expression and decreasing COX-2 and HSP-90 expression within 48 h.

Acknowledgments

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Authors Contribution

BK was involved in concepting the research, supervised the research, writting and reviewing the manuscript. LT performed the research, including processing experimental data, performing analysis, and edited the manuscript. ASN participated in the research design and reviewed the manuscript.

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