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Original Article

Quantification of Active Compounds from *Coffea canephora* Pierre ex A.Froehner cascara and their Potential Against MCF-7 and HeLa

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ABSTRACT

Background: The utilization of coffee cascara, a byproduct of coffee cultivation, in cancer therapy research. This research begins with the rationale of exploring medicinal plants, especially coffee, to obtain compounds that can target cancer cells with fewer side effects. **Objectivity:** This research aims to extract and evaluate the secondary metabolites from robusta coffee cascara, such as friedelin, lupeol, stigmasterol, ursolic acid, caffeine, chlorogenic acid, caffeic acid, and catechin, for their cytotoxic activity against Hela and MCF-7 cells. The aim of this research is also to identify and understand the cytotoxic mechanisms of compounds like stigmasterol, which showed significant cytotoxicity against cancer cells, paving the way for developing targeted cancer therapies from natural sources. **Methods:** Robusta coffee cascara then goes to the process of extraction using ethanol, fractionation, isolation, purification, and characterization, followed by bioactivity evaluation using in vitro method through breast cancer cell line MCF-7 and cervical cancer cell line HeLa and determination of active compound levels. **Results:** The cascara, a byproduct of coffee cultivation, is rich in proteins, polysaccharides, and bioactive compounds. Through extraction and purification processes, eight compounds were isolated and characterized, including **(1)** friedelin, **(2)** lupeol, **(3)** Stigmasterol, **(4)** Ursolic acid, **(5)** caffeine, **(6)** Chlorogenic acid, **(7)** caffeic acid, and (**8)** catechin. Bioactivity evaluation shows that stigmasterol **(3)** is the most cytotoxic compound with a value against Hela cells with an $IC5_0$ value of 25.85 µg/mL in the toxic category and against MCF-7 cells with an IC_{50} value of 12.83 µg/mL in the very toxic category. The results of determining the levels of active compounds in robusta coffee cascara extract showed that friedelin **(1)** 0.539±0.137%; lupeol **(2)** levels were 0.087±0.015%; **(3)** stigmasterol 0.126±0.046%; ursolic acid **(4)** 0.627±0.002%; caffeine **(5)** 3,203±0.069%; chlorogenic acid (**6)** 0.679±0.003%; caffeic acid **(7)** 0.153±0.003% and catechin **(8)** 0.359 0.012% mg/g extract. **Conclusion:** The research on robusta coffee cascara extract as a potential source of anticancer compounds.

Keywords: Cytotoxic, isolation, cascara, Coffea canephora, breast cancer, servical cancer.

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INTRODUCTION

In 2022, Indonesia has become the ninth country that export coffee with a value worth of 1.27 Billion dollars approximately equal to 775.000 metric tons (www.statista.com). There are 2 species known for coffee, which are arabica (*Coffea arabica* L.) and robusta (*Coffea cenephora*), and the latter is the dominant species produced in Indonesia. With the huge amount of coffee consumption, comes the byproduct which approximately 50% will be discarded. During post-harvesting of coffee beans, the farmers would have enormous piles of coffee husk (dried skin, pulp, and parchment part of coffee cherry), known as cascara. Most farmers usually recycle it as fertilized [1] or animal feed[2]. However, due to high levels of caffeine, tannin, and polyphenols, the usage of fertilizer and animal feed has negative impacts such as affecting the animal body weight and less healthy soil. Now, researchers have started to address the possible utilization of cascara as another alternative such as coffee cherry pulp, organic artificial leather, flour, and other food subtitutes[3].

Previous research on the biological activity of robusta coffee cascara has yielded insightful findings. The aqueous extract of robusta coffee cascara demonstrated significant antioxidant capacity, measured using the ORAC method, it showed 8.86 mmol Trolox TE/g and the ABTS method at 3.02 mmol TE/g [4]. Additionally, the cascara extract exhibited antioxidant activity when tested with DPPH resulting in 3.369 mmol TE/g and FRAP methods in 17.513 mmol TE/g[5]. Moreover, the aqueous extract of Arabica coffee cascara exhibits antibacterial activity against various strains, including *Staphylococcus aureus* (MIC 37.5 mg/mL), *Staphylococcus epidermidis* (MIC 4.69 mg/mL), *Pseudomonas aeruginosa* (MIC 75 mg/mL), and *Escherichia coli* (MIC 37.5 mg/mL)[6].

Several research has extensively reported that chlorogenic acid has anticancer activity tested against cancer cells by induced differentiation[7], human breast cancer by inducing apoptosis[8] and even chemopreventive effect [9]. Breast cancer and cervical cancer are among the types of cancer with the highest number of patients. Breast cancer is very common in women, with an estimated 2.1 million cases or about 24.2% of the total cancer cases occurring in women worldwide. Additionally, breast cancer contributes to approximately 15% of the total deaths from cancer in women globally. This indicates that breast cancer has a significant impact on women's health globally.

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Currently, various methods in cancer treatment are being developed, including surgery, targeted therapy, chemotherapy, immunotherapy, hormone therapy, and radiotherapy. Radiotherapy is considered the most affordable treatment method However, the use of radiotherapy is still need a combination with chemotherapy. Chemotherapy remains a commonly used treatment method that involves the use of low molecular weight drugs to damage or limit the growth of cancer cells. In fact, chemotherapy also has negative impacts such as hair loss, nausea, gastrointestinal issues, and the development of treatment resistance. This is because chemotherapy is a combination or regimen of cytotoxic drugs that not only target cancer cells but also normal cells in the body[10].

Over time, researchers continue to develop new findings in cancer treatment, including exploring medicinal plants that containing specific compounds which can effectively target cancer cells with fewer side effects. The challenge of approavhing new cancer drugs with diverse chemical structures and effective combating cancer cells and have low side effects remains a primary focus, emphasizing the exploration of natural sources such as plants (botanical raw materials)[11].

Thus, in this research, we aim to isolate the active compounds from *Coffea canephora* Pierre ex A. Froehner cascara, quantify it, and test its function as an anticancer against Breast cancer cells and cervical cancer cells with lower side effects.

OBJECTIVE

Based on the description above, research will be conducted to quantification of isolated compounds and their potential against MCF-7 and HeLa cancer cell lines.

MATERIALS AND METHODS

The materials used in this research included aluminum sheet kieselgel 60 PF254 0.25 mm (for TLC), silica gel 60 HF254 5–40 µM. The F-bottom 96-well microplate was obtained from Iwaki (Shizuoka, Japan). Absorbances were recorded using a Spectrostar Nano microplate reader (BMG Labtech, Ortenberg, Germany). ^{1}H and ^{13}C-NMR spectra were recorded using a JNM-ECS400 (JEOL Ltd., Akishima, Japan) and Agilent DD2 500 MHz NMR spectrometers (Agilent, Santa Clara, California). Chemicals such as $CeSO_4$, ethanol, n-hexane, ethyl acetate, and EDTA were purchased from Merck, USA. DMEM media, FBS, antibiotic penicillin-streptomycin, trypsin, trypan blue from Gibco, USA. HeLa and MCF-7 cell lines from ATCC, PrestoBlue cell viability reagent from Invitrogen, USA, and cisplatin and DMSO were purchased from Sigma-Aldrich, USA. FT-IR Spectrometer Nicolet iS50, Biosafety Cabinet (BSC) Class II 1300 series type A2, MicroCL17 microcentrifuge, CO2 incubator 8000 DH series, and Microscope (EVOS XL Core) were purchased from Thermo Fisher Scientific (USA). The compounds' mass was recorded using TQD LCMS-Ultra Performance Liquid Chromatography (UPLC) (Waters, USA). 1-D and 2-D NMR 500 MHz Cryo-Probe from Bruker (USA). Cytotoxicity test was carried out using a 96-well plate (Nest Scientific, USA), micropipettes, and tips (Mettler Toledo, USA).

Sample collection

The plant material fruit cascara of *Coffea canephora* Pierre ex A.Froehner was used as plant material. They were taken from Bogor regency, West Java, Indonesia (lat -6.74238°, long 107.006648°), and their identities were verified at Herbarium Bogoriensis, BRIN Cibinong, Indonesia (B 483/V/D1.05.07/10/2021).

Extraction, isolation, purification, and characterization of isolated compounds

Approximately 350 g of extract were soaked in water (2:1) and extracted sequentially using n-hexane, ethyl acetate, and methanol. All the fractions were evaporated using a rotary evaporator and were tested for their cytotoxicity against cervical (Hela cells) and breast cancer cell lines (MCF-7 cells). PrestoBlue™ was used to evaluate its cytotoxicity and Cisplatin was used as a positive control to determine the active fraction[12].

The fractions were subjected to Vacuum Liquid Chromatography (VLC) with silica gel 60 as stationary phase (350 g) and were eluted using gradient polarity of n-hexane: ethyl acetate mixture. Furthermore, the polarity of the mixture was increased using a combination mixture of chloroform and methanol. Each fraction was collected in 100 mL bottles and fractions that had similar patterns of TLC were combined. The VLC fractions thus were subjected to Column Chromatography (CC) using silica gel 70-230 mesh and were eluted with gradient polarity eluent to generate subfractions. These subfractions were further subjected to CC with silica gel 230-400 mesh to generate compounds with high purification. Each potential pure compound was analyzed using TLC with a different type of eluent system.

Friedelin (1): clear crystal; m.p 262-264 °C; ESIMS $[M+H]^+$ m/z 427; ^{1}H NMR ($CDCl_3$, 500 MHz) δH: 1.68 (1H, *dd*, *J* = 5.05 Hz & 12.95 Hz, H-1a), 1.96 (1H, *m*, H-1b), 2.29 (1H, *m*, H-2a), 2.37 (1H, *ddd*, H-2b), 2.23 (1H, *m*, H-4), 1.29 (1H, *m*, H-6a), 1.76 (1H, *m*, H-6b), 1.39 (1H, *m*, H-7a), 1.48 (1H, *m*, H-7b), 1.39 (1H, *dd*, H-8), 1.54 (1H, *m*, H-10), 1.26 (1H, *m*, H-11a), 1.46 (1H, *m*, H-11b), 1.35 (2H, *m*, H-12), 1.29 (1H, *m*, H-15a), 1.43 (1H, *m*, H-15b), 1.35 (1H, *m*, H-16a), 1.54 (1H, *m*, H-16b), 1.56 (1H, *m*, H-18), 1.22 (1H, *m*, H-19a), 1.37 (1H, *m*, H-19b), 1.29 (1H, *m*, H-21a), 1.48 (1H, *m*, H21b), 0.93 (1H, *m*, H-22a), 1.47 (1H, *m*, H-22b), 0.87 (3H, *d*, *J* = 6.70 Hz, H-23), 0.72 (3H, *s*, H-24), 0.86 (3H, *s*, H-25), 1.00 (3H, *s*, H-26), 1.05 (3H, *s*, H-27), 1.18 (3H, *s*, H-28), 0.95 (3H, *s*, H-29), 1.00 (3H, *s*, H-30); 13C NMR ($CDCl_3$, 125 MHz) δH: 22.43 (C-1), 41.68 (C-2), 213.36 (C-3), 58.38 (C-4), 42.29 (C-5), 41.44 (C-6), 18.39 (C-7), 53.25 (C-8), 37.60 (C-9), 59.63 (C-10), 35.78 (C-11), 30.66 (C-12), 39.85 (C-13), 38.45 (C-14), 32.92 (C-15), 36.16 (C-16), 30.15 (C-17), 42.94 (C-18), 35.49 (C-19), 28.32 (C-20), 32.57 (C-21), 39.40 (C-22), 6.97 (C-23), 14.80 (C-24), 18.09 (C-25), 20.41 (C-26), 18.81 (C-27), 32.24 (C-28), 35.17 (C-29), 31.93 (C-30).

Lupeol (2): White amorphous; m.p 215-217°C; ESIMS [M+H]+ m/z 427; 1H NMR (Chloroform-d, 500 MHz) δH: 1.61 (2H, t, H-1), 1.63 (2H, m, H-2), 3.19 (1H, dd,10.8, 5.4, H-3), 0.69 (1H, d, 9.0, H-5), 1.39 (2H, m, H-6), 1.39 (2H, t, 9.0, H-7), 1.27 (1H, t, 5.4, H-9), 1.39 (2H, m, H-11), 1.27 (2H, m, H-12), 1.69 (1H, t, 6.6, H-13) 1.69 (1H, t, 6.6, H-15), 1.52 (1H, m, H-16a), 1.47 (1H, m, H16b), 1.39 (1H, t, 9.0, H-18), 2.38 (1H, m, H-19), 1.92 (1H, m, H-21a), 1.27 (1H, m, H-21b), 1.63 (2H, t, H-22), 0.97 (3H, s, H-23), 0.77 (3H, s, H-24), 0.84 (3H, s, H-25), 1.04 (3H, s, H-26), 0.95 (3H, s, H-27), 0.79 (3H, s, H-28), 4.70 (1H, s, H-29a), 4.57 (1H, s, H-29b), 1.69 (3H, s, H30); 13C NMR (Chloroform-d, 125 MHz) δH: 38.7 (C1), 27.5 (C-2), 78.9 (C-3), 38.9 (C-4), 55.3 (C-5), 18.3 (C6), 34.3 (C-7), 40.8 (C-8), 50.4 (C-9), 37.1 (C-10), 20.9 (C-11), 25.2 (C-12), 38.1 (C-13), 42.9 (C-14), 27.4 (C-15), 35.6 (C-16), 48.2 (C-17), 48.3 (C-18), 47.9 (C-19), 150.9 (C-20), 29.9 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.6 (C-27), 18.0 (C-28), 109.4(C-29), 19.3 (C-30). (Elya, et al., 2024).

Stigmasterol (**3**): Colorless crystal; m.p 134-136°C; ESIMS [M+H]+ m/z 413; 1H NMR (Chloroform-d, 500 MHz) δH: 1.84 (2H, m, H-1), 1.51 (2H, m, H-2), 3.51 (1H, m, H-3), 2.24 (2H, dd, 10.5, 2.1, H-4), 5.34 (1H, d, 5.4, H-6), 1.84 (2H, m, H-7), 1.93 (1H, m, H-8), 1.44 (1H, m, H-9), 1.47 (2H, m, H-11), 2.00 (2H, dd, 9.3, 3.0, H-12) 1.10 (1H, m, H14), 1.55 (2H, m, H-15), 1.25 (2H, m, H-16), 1.07 (1H, m, H-17), 0.68 (3H, s, H-18), 0.99 (3H, s, H-19), 1.99 (1H, m, H-20), 0.91 (3H, d, 6.3, H-21), 4.99 (1H, dd, 15.2, 8.7, H-22), 5.14 (1H, dd, 15.2, 8.7, H-23), 1.93 (1H, m, H-24), 1.65 (1H, m, H-25), 0.81 (3H, d, 6.5, H-26), 0.79 (3H, d, 6.5, H-27), 1.25 (2H, m, H-28) , 0.84 (3H, t, 3.3, H-29); 13C NMR (Chloroform-d, 125 MHz) δH: 37.0 (C-1), 31.7 (C-2), 71.5 (C-3), 42.0 (C-4), 140.5 (C-5), 121.5 (C-6), 31.4 (C-7), 31.6 (C-8), 49.9 (C-9), 36.3

(C10), 20.9 (C-11), 39.5 (C-12), 42.1 (C-13), 56.5 (C-14), 24.1 (C-15), 28.0 (C-16), 55.8 (C-17), 11.6 (C-18), 18.8 (C-19), 40.3 (C-20), 18.6 (C-21), 138 (C-22), 129.0 (C23), 51.0 (C-24), 28.9 (C-25), 19.6 (C-26), 19.2 (C-27), 22.8 (C-28), 11.7 (C-29). S (Elya, et al., 2024).

Ursolic Acid (4): White amorphous; m.p 215-217°C; ESIMS [M+H]+ m/z 457; 1H NMR (DMSO, 500 MHz) δH: 1.0 (1H, 13.15, H-1a), 1.52 (1H, m, H-1b), 1.45 (2H, q, H-2), 3.00 (1H, m, H-3), 4.29 (1H, d, OH-3), 0.66 (1H, br-s, H-5), 1.45 (1H, m, H-6a), 1.29 (1H, m, H-6b), 1.42 (1H, m, H7a), 1.26 (1H, m, H-7b), 1.44 (1H, t, 6.8, H-9), 1.85 (2H, t, 3.0, H-11), 5.13 (1H, t, 3.25, H-12), 1.80 (2H, m, H-15) 1.91 (1H, m, H-16a), 1.52 (1H, m, H-16b), 2.10 (1H, d, 11.25, H-18), 1.52 (1H, m, H-19), 1.28 (1H, m, H-20), 1.42 (2H, m, H-21), 1.54 (2H, m, H-22), 0.89 (3H, s, H23), 0.68 (3H, s, H-24), 0.87 (3H, s, H-25), 0.75 (3H, s, H-26), 1.04 (3H, s, H-27), 11.93 (1H, s, OH-28), 0.80 (3H, d, 6.45 H-29), 0.89 (3H, d, H-30); 13C NMR (DMSO, 125 MHz) δH: 39.2 (C-1), 28.2 (C-2), 78.2 (C3), 39.6 (C-4), 55.9 (C-5), 18.8 (C-6), 33.7 (C-7), 40.1 (C8), 48.1 (C-9), 37.5 (C-10), 23.7 (C-11), 125.7 (C-12), 139.3 (C-13), 42.6 (C-14), 28.8 (C-15), 25.0 (C-16), 48.1 (C-17), 53.6 (C-18), 39.5 (C-19), 39.4 (C-20), 31.1 (C-21), 37.4 (C-22), 28.8 (C-23), 16.5 (C-24), 15.7 (C-25), 17.5 (C-26), 24.0 (C-27), 179.7 (C-28), 17.5 (C-29), 21.4 (C30). (Elya, et al., 2024).

Caffeine (5): White amorphous; m.p 190-192 °C; ESIMS $[M+H]^+$ m/z 195; ^{1}H NMR (D_2O, 500 MHz) δ_H: 7.87 (1H, *s*, H-8), 3.23 (3H, *s*, H-10), 3.40 (3H, *s*, H-11), 3.88 (3H, *s*, H-12); ^{13}C NMR (D_2O, 125 MHz) δ_C: 154.84 (C-2), 150.69 (C-4), 109.99 (C-5), 158.37 (C-6), 146.17 (C-8), 30.61 (C-10), 32.51 (C-11), 36.16 (C-12).

Chlorogenic Acid (6): White crystal; m.p 134-136 °C; ESIMS $[M-H]^-$ m/z 353; ^{1}H NMR (D_2O, 500 MHz) δ_H: 2.15 (1H, *dd*, *J* = 3.25 Hz & 15.05 Hz, H-2a), 2.06 (1H, *t*, *J* = 3.15 Hz, H-2b), 4.26 (1H, *q*, *J* = 3.3 Hz, H-3), 3.86 (1H, *dd*, *J* = 3.35 Hz & 9.95 Hz, H-4), 5.31 (1H, *m*, H-5), 2.20 (1H, *dt*, *J* = 2.85 Hz, H-6a) 2.03 (1H, *m*, H-6b), 7.07 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.87 (1H, *d*, *J* =8,2 Hz, H-5'), 7.00 (1H, *dd*, *J* = 2.1 Hz & 8.25 Hz, H-6'), 7.52 (1H, *d*, *J* = 15.95 Hz, H-7'), 6.26 (1H, *d*, *J* = 16.0 Hz, H-8'); 13C NMR (D_2O, 125 MHz) δ_C: 78.49 (C-1), 38.92 (C-2), 72.35 (C-3), 74.51 (C-4), 72.74 (C-5), 40.07 (C-6), 182.55 (C-7), 128.32 (C-1'), 116.58 (C-2'), 145.95 (C-3'), 149.04 (C-4'), 117.77 (C-5'), 124.34 (C-6'), 147.72 (C-7'), 115.91 (C-8'), 170.75 (C-9').

Caffeic Acid (7): White amorphous; m.p 235-236°C; ESIMS [M+H]+ m/z 181; UV λmax (MeOH) nm (log ε): 203.53 and 340.51 nm; 1H NMR (DMSO, 500 MHz) δH: 6.98 (1H, d, 2.0 Hz, H-2), 6.71 (1H, d, 8.16 Hz, H-5), 6.92 (1H, dd, 8.16, 2.12 Hz, H-6), 7.37 (1H, d, 15.88 Hz, H-7), 6.12 (1H, d, 15.92 Hz, H-8), 12.06 (1H, s, 9-OH), 9.47 (1H, s, 3-OH), 9.07 (1H s, 4-OH); 13C NMR (DMSO, 125 MHz) δH: 126.22 (C-1), 115.16 (C-2), 146.08 (C-3), 148.66 (C-4), 116.26 (C-5), 121.67 (C-6), 145.12 (C-7), 115.63 (C-8), 168.42 (C-9). See Fig. S4. (Elya, et al., 2024).

Catechin (8): Pale white amorphous; m.p 175-177 °C; ESIMS $[M-H]^-$ m/z 289; ^{1}H NMR (MeOD, 500 MHz) δ_H: 4.56 (1H, *d*, *J* = 7.5 Hz, H-2), 3.97 (1H, *q*, *J* = 7.9 Hz, H-3), 2.84 (1H, *dd*, *J* = 5.4 Hz & 16.1 Hz, H-4a), 2.50 (1H, *dd*, *J* = 8.1 Hz & 16.1 Hz, H-4b), 5.85 (1H, *d*, *J* = 2.3 Hz, H-6), 5.92 (1H, *d*, *J* = 2.3 Hz, H-8), 6.83 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.76 (1H, *d*, *J* = 8.1 Hz, H-5'), 6.71 (1H, *dd*, *J* = 2.0 Hz & 8,1 Hz, H-6'); ^{13}C NMR (MeOD, 125 MHz) δ_C: 82.84 (C-2), 68.81 (C-3), 28.53 (C-4), 156.92 (C-5), 95.25 (C-6), 157.59 (C-7), 96.25 (C-8), 157.83 (C-9), 100.79 (C-10), 132.19 (C-1'), 115.23 (C-2'), 146.25 (C-3'), 146.23 (C-4'), 116.08 (C-5'), 120.06 (C-6').

Culture cell conditions and cytotoxic assay

The cytotoxicity of compounds **1–8** was determined with a cell viability test using PrestoBlue® assay. The cells were maintained in a Roswell Park Memorial Institute (RPMI) medium with 10% (v/v) Fetal Bovine Serum (FBS) and 1 µL/1 mL antibiotics (1% penicillin-streptomycin). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 (Aisyah et al., 2022; Nur et al., 2021). MCF-7 cells were plated in 96 multiwell culture plates at a density of 1.7×10^4 cells/well. After twenty-four hours, the medium was discarded, and fresh medium containing samples (*C. canephora* extracts, fractions, and isolates) with different concentrations of 7.81, 15.63, 31.25, 62.50, 125.00, 250.00, 500.00, 1000.00 µg/mL and with the positive control cisplatin. After incubation with the sample for 24 h, PrestoBlue® reagent (resazurin dye) was added. The PrestoBlue® assay results were read using a multimode reader at 570 nm (Tecan Infinite M200 PRO, Switzerland). The IC_{50} value was determined by linear regression using Microsoft Excel software. Then, the IC_{50} value was calculated as the extract concentration that inhibited cell viability by 50% after the exposure time. The selectivity index (SI) was defined as the ratio of the IC_{50} value observed against cancer cell lines to the IC_{50} value observed in non-malignant cells[28].

Cytotoxicity assay by reduction of Resazurin Method

This test was performed as described by Sianipar et al (2020) [12].

Preparation of media, sample, and positive control

The liquid culture media DMEM (Dulbecco's Modified Eagle Medium) containing 10% Fetal Bovine Serum (FBS) and 0,001% antibiotic, positive control cisplatin, and a sample at a certain concentration was prepared. The solvent used in this study is 2% DMSO.

Preparation of cells

The tested cells (Hela and MCF-7 cell line) were thawed at 37°C in a water bath. 1 mL of RPMI media was added, then the suspension was put in the sterile microtube, and spun at 3000 rpm for 5 minutes. The supernatant was discarded and added with 1 mL of media, resuspended, and placed in a cell culture dish that was prefilled with 3 mL of media. The cell's morphology was observed with an inverted microscope, and incubated at 37°C in the 5% CO2 incubator for 24h or until a minimum of 70% confluent.

Inoculation of cells in a 96-well plate

Cells were rinsed with PBS, added with 1 mL of Trypsin n-EDTA, and incubated for 5 minutes at 37°C. Cells were put into a prefilled 0.5 mL of media in a microtube and centrifuged at 3000 rpm. Pellets were resuspended with 1 mL of media RPMI. Cell count was calculated using trypan blue (1:1) until it reached 17.000 cells/well, then cell culture was put on a 96-well plate, and incubated for 24 h (or until minimum 70% confluent) at 37°C in 5% CO2.

Cell treatment with sample and control

Samples (extract, fraction, and isolate) were diluted into 8 serial dilutions (500; 250; 125; 62,50; 31,25; 15,63; 7,81; 3,91 µg/ml) using media and as positive control cisplatin were used. A 96-well plate prefilled with cells was labeled. The media from each well were discarded. Then, 100 µL of samples and positive control were added to the well and reintubated for 48 h. Next, it was added with 100 µL of Prestoblue and incubated for 2 h and was measured at 570 nm (resofurin) and 600 nm (resazurin) using the multimode reader. The absorbance was calculated at the defined concentration:

Y = (abs 570 – abs 600) X media cell

Y or corrected absorbance = absorbance of samples at the define concentration

Abs 570 = absorbance of samples at the defined concentration measured at 570 nm

abs 600 = absorbance of samples at defined concentration measured at 600 nm

X media cell = average absorbance of the media

Determination of Cytotoxicity of the samples against Hela and MCF-7 cells using reduction resazurin method

Each fraction, isolate, and extract of robusta coffee cascara was tested against Hela and MCF-7 cells. PrestoBlueTM was used to investigate the viability of the cells. This method is based on the changing blue color of Resazurin to the pink color of resofurin [29,30].

HPLC quantitative analysis of 1-8 in Coffea canephora Pierre ex A.Froehner cascara extract

Preparation of reference standard solution

The standard (**1-8**) solution (1 mg/mL) was freshly prepared in MeOH. The resulting solution was sonicated for 2 min and then filtered through 0.22 µm PTFE filter assemblies.

Liquid chromatography-mass spectrometry analysis

- Instrumentation.

Analysis was performed on an HPLC PDA (Waters, Milford, Michigan, USA) with Reliant® T3 C18 (150 x 4.6 mm, 5 µm) as a column for separation.

- Liquid chromatography conditions.

The mobile phase consisted of solvent A: Water +0.1% formic acid and solvent B: MeCN +0.1% formic acid. The elution was performed in gradient mode during a time course of 15 min as follows: 0–0.5 min, 10–20% B; 0.5–3.0 min, 20–28% B; 3.0–4.0 min, 28–30.3% B; 4.0–5.0 min, 30.3–33.3% B; 5.0–5.5 min, 33.3–50% B; 5.5–6.5 min, 50–70% B; 6.5–7.0 min, 70–80% B; 7.5–8.5 min, 80–100% B; 8.5–15.0 min, held to 100% B at flow rate 0.3 mL/min. Finally, the composition was returned to the initial (10% B) in 0.01 min. The column was equilibrated for 5 min before the next injection. The column was operated at 40 °C and the sample/standard injection volume was 1 µL.

Validation method

Validation of the proposed method was performed by carrying out the required validation parameters, including linearity, limit of detection (LOD), and limit of quantification (LOQ).

- Linearity

Several concentration series of **1-8** were prepared and analyzed using HPLC. Each concentration was analyzed three times (triplication). Linear least squares regression was used to calculate the slopes, intercepts, and correlation coefficients. The acceptance requirement is the value of the correlation coefficients ≥0.9950.

- Limit of detection (LOD) and limit of quantification (LOQ)

The LODs and LOQs were defined as the lowest concentrations of standard solutions or as the lowest spiked concentrations in fortified samples that produce a signal-to-noise ratio of 3 and 10, respectively. The calculation of the detection limit and quantification limit uses the following formula:

$$LOD = 3.3\ x\ \frac{\mathrm{SD}}{a}\ (1)$$

$$LOQ = 10\ x\ \frac{\mathrm{SD}}{a}\ (2)$$

where: SD = Standard Deviation

a = The slope of the calibration curve.

Sample preparation

Each sample mass of 40 mg was added into 0.8 mL of MeOH, and a sonicator was used to help optimize the dissolving process. The insoluble material was separated with centrifugation at 4000 rpm for 5 min. The supernatant (soluble material) was subsequently filtered using a 0.22 µm Millipore membrane filter. The filtrate (100 µL) was dissolved in deionized water to make 1 mL solution for the analysis with HPLC using the validated method.

RESULTS AND DISCUSSION

In this study, we successfully isolated 8 compounds from Coffea *canephora Pierre ex A.Froehner* cascara which are 4 steroids (Friedelin, Stigmasterol, Ursolic acid, Lupeol), 1 alkaloid (Caffein) and 3 phenolic compounds (chlorogenic acid, caffeic acid, and catechin). The yield of caffeine isolated from the Robusta cascara (3.2%) is slightly higher than that of the coffee bean itself (0.3-2.7%) although it depends on several aspects such as the brewing technique, plantation area, roasting, and time of extraction, etc.

Caffeine and the three phenolic compounds are also found as major bioactive compounds in the coffee bean itself. These compounds were tested and showed that they have an effect in reducing human colorectal cancer cell lines via cell phase arrest and apoptosis (reviewed in Buldak et al, (2018)[31]).

Friedelin (1) isolated from Robusta coffee cascara is 0.5% mg/g dry weight determined with HPLC, which is lower than that of isolated from *Quercus suber L* but much higher than found in *Putranjiva roxburghii Wall* and *Maytenus ilicifolia* (Table 5.). Friedelin has gained interest in pharmaceutical research due to his unique biological ability such as anticancer. The cytotoxicity of friedelin measured in this study showed that it is comparable to cisplatin (Table 2.). This result is similar to friedelin isolated from the stem of *Ficus drupacea* L. [32] against the MCF-7 cancer cell line.

Ursolic acid (4) level content isolated from *Ilex aquifolium* L. leaves (1.3%) is 2-fold higher than that of robusta coffee (0.62% mg/g) (Table 5.). The highest amount of ursolic acid was found in *Calendula officinalis* (20 mg/g) and coffee arabica (18 mg/g)[33,34]. However, in terms of anticancer activity, ursolic acid showed the highest cytotoxicity compared to the other 7 compounds isolated (IC_{50} 12.83 µg/mL) and even higher than that of cisplatin (IC_{50} 25.87 µg /mL) as positive control against MCF-7 cancer cell lines. This result is in line with the finding of Ali et al., (2019)[35] who isolated ursolic acid from the fruit extract of *Paulownia tomentosa*. The ursolic acid of *Paulownia tomentosa* has IC_{50} 3.5 µg /mL and also showed much higher cytotoxicity compared to Doxorubicin (26,1 µg /mL) against MCF-7 cancer cell lines. Furthermore, its cytotoxicity also showed strong activity against Hela Cells.

In comparison with Silva et al., (2020)[36] who isolated **chlorogenic acids (6)** from arabica coffee husk, the chlorogenic acid found in robusta coffee cascara is much higher 0.67% mg/g versus 16.64-337 µg /g but lower than that found in Potato skin. The highest level of chlorogenic acid isolated is from the flowering bud of Lonicera japonica 98.96 mg/g (reviewed in Gupta et al., (2022)[37]). Chlorogenic acid possesses a wide range of pharmacological properties such as antibacterial hepatoprotective, antimicrobial, immunomodulatory, antioxidant, antidiabetic, and anticancer activities. It has been reported that chlorogenic acid is an anticancer by inhibiting the cell cycle, inducing apoptosis, and suppressing the proliferation of cancer cells. Its cytotoxicity against MCF-7 cancer cell lines showed a comparable to cisplatin (Table 2.). Several reports showed the cytotoxicity level against MCF-7 widely varied from 50-500 µM, and that chlorogenic acid behaves in a time and dose-dependent manner.

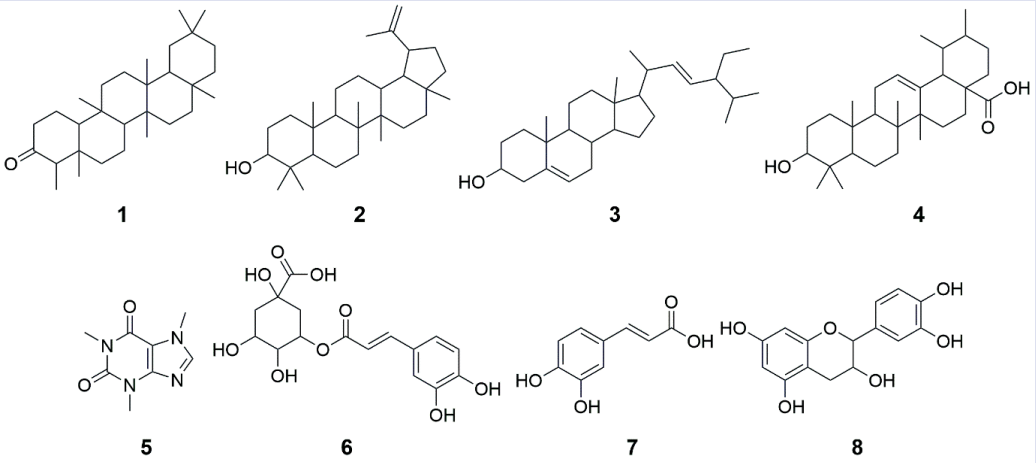

Figure 1. Structures of compounds 1-8.

Table 1. Distribution of compound 1-8 from many sources.

Compound	Species (part)	Reference
Friedelin (**1**)	*Maytenus ilicifolia*	13
	Croton ferrugineus var. elegans (Kunth) (Leaves)	14
Lupeol (**2**)	*Tamarindus indica linn*	15
	Brassica oleracea L	16
Stigmasterol (**3**)	*Ophiopogon japonicas*	17
	Annonamuricata L. (leaf)	[18
Ursolic Acid (**4**)	*Eriobotrya fragrans*	19
	Catharanthus roseus	20
Caffeine (**5**)	*Green tea leaves*	21
	Coffee Husk	22
Chlorogenic Acid (**6**)	*Lonicera macranthoides*	23
	Solanum tuberosum	24
Caffeic Acid (**7**)	*Solanum tuberosum*	25
	Carrot	26
Catechin (**8**)	*Camellia sinensis*	27

Table 2. Results of in vitro cytotoxicity assay.

Compounds	IC_{50} (µg/mL) ± SD	
	Hela Cells	MCF-7 Cells
1	63.74 ± 0.21	30.15 ± 0.11
2	85.35 ± 0.40	74.69 ± 0.48
3	25.85 ± 0.17	12.83 ± 0.15
4	84.70 ± 0.53	64.89 ± 0.13
5	188.71 ± 0.46	200.21 ± 0.60
6	140.19 ± 0.23	32.35 ± 0.13
7	124.77 ± 0.26	114.44 ± 0.50
8	30.46 ± 0.12	66.21 ± 0.14
Cisplatin (+control)	19.85 ± 0.14	25.87 ± 0.17

Table 3. Validation parameters of the analytical method; linearity, and sensitivity.

Compound	Sensitivity				
	Linearity Equations	(R^2)	Linear Range (mg/L)	LOD (mg/L)	LOQ (mg/L)
1	*y = 1040x – 2594.7*	0.9997	6.25 – 200	0.1748	0.5297
2	*y = 7400.2x – 8168.6*	0.9998	6.25 – 200	0.5579	1.6906
3	*y = 7087.7x – 9665.9*	0.9997	6.25 – 200	0.5261	1.5941
4	*y = 4182.4x – 5172.1*	0.9996	6.25 – 200	0.2026	0.6139
5	*y= 19572x - 53262*	0.9997	1.95 – 250	0.002	0.006
6	*y = 16237x - 18016*	0.9999	1.95 – 250	0.006	0.019
7	*y = 31419x - 49335*	0.9999	1.95 – 250	0.036	0.109
8	*y = 72337x + 35283*	0.9998	1.95 – 250	0.012	0.039

Table 4. Compound 1-8 content in extract using HPLC.

Compound	Replication	Sample (mg)	AUC	content (%) (mg/g)	Average content (%)± SD
1	1	42.1	8293	0.551	
	2	42.4	8363	0.502	0.530 ± 0.137
	3	42.1	8567	0.537	
2	1	42,0	7343	0.090	
	2	42.1	7235	0.085	0.087 ± 0.015
	3	42.1	7471	0.087	
3	1	41.6	7341	0.150	
	2	42.1	7561	0.116	0.126 ± 0.046
	3	42.1	6925	0.111	
4	1	42.1	45615	0.578	
	2	42.4	57242	0.622	0.627 ± 0.002
	3	42.1	54515	0.679	
5	1	45.0	2434782	3.178	
	2	44.0	2412914	3.150	3.203 ± 0.069
	3	45.2	2516467	3.282	
6	1	45.0	422541	0.678	
	2	44.0	421632	0.676	0.679 ± 0.003
	3	45.2	426037	0.683	
7	1	45.0	144405	0.154	
	2	44.0	138675	0.149	0.153 ± 0.003
	3	45.2	148677	0.157	
8	1	45.0	1046810	0.349	
	2	44.0	1061627	0.354	0.359 ± 0.012
	3	45.2	1115903	0.373	

Table 5. Quantification compounds isolated from different species.

Compounds	Species	Quantification	Reference
Friedelin	*Putranjiva roxburghii* Wall	Leaves extract (0.003% *w/w*) and (0.04% w/w)	[41]
	Quercus suber L.	Cork 2.47 g/kg dry weight	[42]
	Maytenus ilicifolia	0.44 mg /L	[43]
Ursolic acid	*Saurauja roxburghii* Wall	Leaves extract 1.4 mg/kg	[44]
	Ilex aquifolium L	Leaf extract 1.3% dried leaves	[45]
	Calendula officinalis	20.5 mg/g	[46]
	Coffea arabica	18 mg/g	[33]
	Coffee robusta (stem)	19.8 mg/kg dry weight	[34]
Chlorogenic acid	*Coffea arabica*	Coffee bean 4.67–5.87% dry	[47]
	Coffee Husk	16.64 to 337.07 µg/g	[36]
	Coffee silverskin	1.06 g/kg - 2.68 g/kg	[48]
	Potato skin	1.3–4.1 mg/g dw	[49]
	Hylocereus undatun	1.30 µg/g (peel) 0.55 µg/g (pulp)	[50]
Lupeol	*Hygrophila schulli*	5.02±0.2 % w/w on dry weight	[51]
	Andrographis echioides	0.265 µg/100 mg	[52]
	Betula alnoides	3,45% w/w dry	[53]
	Swertia chirata	2.98 ± 0.02 % AE	[54]
	Costus igneus	473 µg/100 mg dw	[55]
Stigmasterol	*Viola odorata*	0.007 µg/mL in EE	[56]
	Cyperus esculentus	7,58% ethanolic extract	[57]
	Dipteracanthus patulus (Jacq.) Nees	0,22 mg/g dw	[58]
	Costus Igneus	1913 µg/100 mg dw	[55]
Caffeine	*Camellia crassicolumna*	37.17 ± 37 mg/g dw	[59]
	Camelia sinensis	89.02 ± 0.42 mg/g dw	[60]
Caffeic acid	*Hylocereus undatun*	108.94 µg/g (peel) 31.78 µg/g (pulp)	[50]
	Lactuca virosa L.	1.127 ± 0.030 % dw	[61]
Catechin	*Camelia sinensis*	1.25 ± 0.54 mg/g dw	[60]

Table 6. Anticancer activity of isolated compounds from different species.

Compounds	Isolated from	Cell Lines	Activity	References
Fridelin	*Wedelia trilobata*	B-cell lymphoma-2 (Bcl-2)	In silico, molecular docking. Has the lowest energy binding	63
	Ficus drupacea L. stem	MCF-7 Breast cancer	IC_{50} of 22.81 ± 2.1 µg/mL	32
	Elaeocarpus floribundus Blume	HeLa cervical cancer line	IC_{50} of 3.54 ± 0.30 µg/mL	64
	Ficus drupacea L. (stem)	HeLa cervical cancer line	IC_{50} of 20.42 ± 2.3 µg/mL	32
	Cassia tora	HeLa cervical cancer line	IC_{50} of 19.3 ± 1.27 µg/mL	65
Ursolic acid	*Saurauja roxburghii* Wall	C6 rat glioma and	IC_{50} of 10–100 µM	44
	Paulownia tomentosa	HEPG2, A-549, MCF-7	IC_{50} of 14.5, 68.4, 3.5 (µg/ml)	35
Chlorogenic acid	*n.d*	Human NCI-H446, Huh7, Bel-7402, HEK293T, HCT-116, U87MG, and M059J	Reduced proliferation rate, migration/invasion ability, and mitochondrial ATP production.	66
	n.d	Human colon cancer cell line HCT116 and HT29	Inhibit cell viability	67
	Nerium oleander L.	Caco-2 and HT29	Inhibits the proliferation of human colon cancer cells, involving cell cycle arrest and apoptosis	68
Lupeol	*Avicennia marina*	Breast cancer cell line MCF-7 and Liver cancer cell line Hep3B	Inhibit the growth of MCF-7 and inducing apoptosis on Hep3B	69
	Grewia lasiocarpa	HEK293, HeLa, and MCF-7 cell lines.	Inducing apoptosis in each cells	70
	Polyalthia lateriflora	Breast cancer cell line MCF-7	IC_{50} of 42 µg/mL	71
Stigmasterol	*Typhonium flagelliforme*	Breast cancer cell line MCF-7	IC_{50} of 0.1623 µM	72
	Abrus precatorius	Breast cancer cell line MDA-MB-231	IC_{50} of 74,2 µg/mL	73
	Aglaia simplicifolia	Cervical cancer cell line HeLa	IC_{50} of 26,42 µg/mL	74
Caffeine	*n.d.*	Human cervical cancer cell line HeLa	Caffeine-assisted chemotherapy enhances the antiproliferation effect of cisplatin and lowered the $IC5_0$ of cisplatin from 8.93 µM to be 2.75 µM	75
Caffeic acid	*Coffee sp.*	Hep3B cell line	Induced apoptosis: TRAIL-mediated apoptosis via CHOP-induced death receptor 5 upregulation	40
	Perilla frutescens	Hepatocellular carcinoma HepG2	Induced apoptosis through the positive regulation of DR5 via p38	39
	n.d	Breast cancer cell line MCF-7	Induced apoptosis with IC_{50} of 159 µg/mL	38
Catechin	Green tea leaves	Breast cancer cell line MCF-7	Induce Inhibition of PTP1B Phosphatase in Breast Cancer Cell MCF-7	76
	Green tea leaves	Hepatocarcinoma cell HepG2	Inducing apoptosis in HepG2 cells via caspase dependent pathway	77
	Kenyan tea extracts	Human cervical cancer cell line HeLa	Hindering the proliferation of cervical cancer cells and inducing appoptosis by blocking the interaction of HPV16E6 with host proteins	78

Caffeic acid (7) has been successfully isolated and the total average value in the extract of robusta coffee cascara is 0.153 ± 0.003 mg/g. This value is 14.16% higher compared to caffeic acid content in *Hylocereus undatus*, which is 108.94 µg/g (peel) and 31.78 µg/g (pulp) (Table 5.). The caffeic acid isolated from robusta coffee cascara exhibits cytotoxic properties against breast cancer MCF-7 cells and cervical cancer HeLa cells with IC_{50} values of 14.44 ± 0.50 and 124.77 ± 0.261 µg/mL, respectively. These values are considered moderate cytotoxic compounds. The value is also higher compared to the study by Rezaei-Seresht et al., (2019) using Caffeic acid against the same cells, namely MCF-7. The results obtained turned out to be 36% higher [38]. Besides showing significant potential against breast and cervical cancer cells, this caffeic acid compound also has substantial potential, as shown in studies by Dilshara et al., (2016) and Yang et al., (2013), which found that caffeic acid isolated from *Coffee sp*. and *Perilla frutescens* has potential in combating and dealing liver cancer cells or hepatocarcinoma cells through the mechanism of cell apoptosis .

Stigmasterol (3) isolated from robusta coffee cascara exhibits the strongest cytotoxic activity against breast cancer cells MCF-7 and Hela amongst all isolated compounds, with IC_{50} values of 12.83 ± 0.15 and 25.85 ± 0.17 µg/mL, respectively (Table 2). Although the concentration of stigmasterol in robusta coffee cascara (0.126 ± 0.046 mg/g dw) is lower compared to that isolated from other species as reported by Manjula et al. (2013) (191.3 mg/g dw from Costus igneus plants)[55], the high cytotoxic activity of this compound indicates significant potential in the development of plant-based cancer therapy, particularly in addressing breast cancer.

The anti-cancer effect and cytotoxic activity of stigmasterol, a compound derived from robusta coffee cascara, has garnered attention due to its multifaceted mechanisms of action. Studies have indicated that stigmasterol's efficacy in combating cancer is attributed to its ability to inhibit sphingosine kinases, which play a crucial role in cell survival and proliferation[62]. Additionally, stigmasterol has been found to stabilize tubulin, a key component of the cell's cytoskeleton involved in cell division. By inhibiting sphingosine kinases and stabilizing tubulin, stigmasterol induces cell death and growth arrest specifically in breast cancer cells. This dual mechanism underscores the therapeutic potential of stigmasterol as a targeted agent in cancer treatment, highlighting its intricate role in disrupting cancer cell pathways and promoting anti-cancer effects[62].

Caffeine (5) was isolated in the highest amount as dry weight, confirmed using HPLC instrumentation, with a quantity of 3.203 ±

0.069 mg/g dw. Caffeine is indeed a major compound found in coffee plants. However, the abundance of this major compound does not align with its anticancer properties against breast cancer cells MCF-7 and cervical cancer cells HeLa, as shown in (Table 2). Both against MCF-7 and HeLa, caffeine exhibits unfavorable values, with IC_{50} values of 200.21 ± 0.060 and 188.71 ± 0.46 µg/mL, respectively. Structurally, this compound does not possess cytotoxic activity. However, research conducted by Lu et al. (2014) explored Caffeine-assisted chemotherapy, where caffeine and cisplatin were combined and used to induce the human cervical cell line HeLa. The results indicated that this combination or regimen enhances the antiproliferation effect of cisplatin and lowers the IC50 of cisplatin from 8.93 µM to 2.75 µM[75].

According to the findings of Lu et al., (2014) caffeine introduces a new regulatory process involving the SR splicing factor SRSF3, which modifies the isoform status of p53. Caffeine was observed to reduce the expression of SRSF3 in a manner dependent on dosage, resulting in the alternate transcription of the p53 isoform instead of its counterpart. While increasing the amount of SRSF3 did not reverse the alternate splicing of the p53 gene, our data corroborated that caffeine spurred alternative splicing in other genes targeted by SRSF3, such as pyruvate kinase M1 and 2, or HIF-1¸ and HIF-2¸ pre-mRNA. Recent studies also indicate that caffeine prompts SC35 production in HeLa cells, influencing a distinct set of genes [75].

Lupeol (2) is a compound with moderate cytotoxic activity against breast cancer cells MCF-7 and HeLa, measured by IC_{50} values of 74.69 ± 0.48 and 85.35 ± 0.40 µg/mL, respectively. Although its cytotoxic activity is not as strong as other compounds like stigmasterol, lupeol still shows potential as an anticancer agent. However, its concentration in robusta coffee cascara is relatively low, at around 0.087 ± 0.015 mg/g dw (Table 4). This indicates that while lupeol has sufficient cytotoxic activity, its limited availability in robusta coffee cascara may be a consideration in the development of plant-based natural compound therapies for cancer.

Based on (Table 6.), friedelin indeed has good cytotoxic activity against breast cancer cells MCF-7 and HeLa. According to Subash-babu et al., (2017), friedelin and other types of steroids and terpenoids have a good IC_{50} value ranging from 1-100 µg/mL because these compounds can induce apoptosis in MCF-7 cells by upregulating tumor suppressor genes and activating p53 and caspases [79].

CONCLUSION

This study highlights stigmasterol as the most potent cytotoxic compound found in robusta coffee cascara, exhibiting significant activity against Hela cells (IC_{50} value of 25.85 µg/mL) and MCF-7 cells (IC_{50} value of 12.83 µg/mL). Analysis of active compound levels in the extract revealed varying concentrations of compounds such as friedelin, lupeol, stigmasterol, ursolic acid, caffeine, chlorogenic acid, caffeic acid, and catechin, with caffeine showing the highest concentration. Notably, compounds like friedelin, ursolic acid, and lupeol were identified for the first time in the cascara part of robusta coffee, expanding our understanding of its chemical composition. These findings underscore the potential of robusta coffee cascara as a valuable source of bioactive compounds, particularly stigmasterol, in the pursuit of novel anticancer therapies.

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