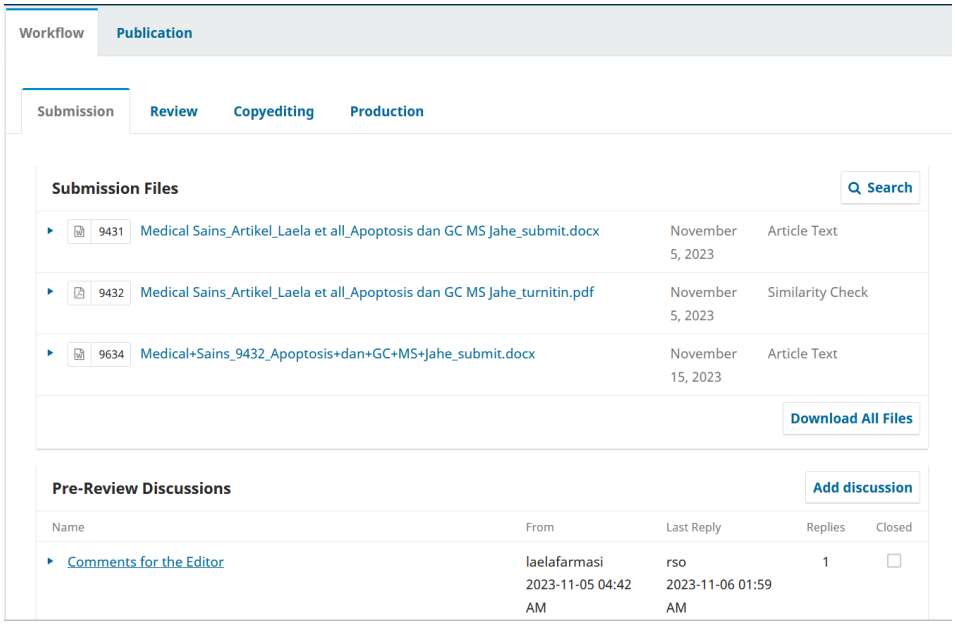
5 November 2023, submit di OJS:



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Cover Letter_Medical sains

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6 November 2023

Participants

Rinto Susilo (rso)

Laela Hayu Nurani (laelafarmasi)

Messages

Note	From
Dear Authors, After we have reviewed your research using plants as test material, please attach a certificate of plant determination, the file can be attached/uploaded in the reply to this discussion column. Thank You Best regards Rinto Susilo	rso 2023-11-06 02:03 AM
Dear Dr. Rinto Susilo, Thank you for the review. Regarding plant determination, a letter of determination is attached. Thank you very much. Laela Determinasi_tanaman jahe.pdf	laelafarmasi 2023-11-06 02:12 AM

Add Message

[MS] New notification from Medical Sains : Jurnal Ilmiah Kefarmasian

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Rinto Susilo <jurnalstfmc@gmail.com> Mon, Nov 6, 2023, 9:00 AM

to me

You have a new notification from Medical Sains : Jurnal Ilmiah Kefarmasian:

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RINTO SUSILO

Rinto Susilo

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10 Desember 2023

[MS] Editor Decision External Inbox ×

Fajar Aji Lumakso <jurnalstfmc@gmail.com> Sun, Dec 10, 2023, 6:57 PM

to me, Citra, Siti, Dwi, Any

Laela Hayu Nurani, Citra Ariani Edityaningrum, Siti Rofida, Dwi Utami, Any Guntarti:

We really appreciated the preparation of this article which will give impact on knowledge. Based on reviewer's comment, we have reached a decision regarding your submission to Medical Sains : Jurnal Ilmiah Kefarmasian, "Apoptosis Induction of Zingiber oficinale Ethanol Extract in HeLa Cells and GC/MS Profile".

Our decision is: Revisions Required
Below are the comments from reviewer

Dear Author,

Regards,
Thank you for submitting a manuscript with a topic, but there are some recommendations that must be considered so that this manuscript is worthy of publication.
1. Language quality is improved as suggested
2. in the abstract, you need to add details of the article being reviewed
3. in the introduction, clearly state the objectives
4. In the method, it must be rearranged; see comments on the manuscript
5. In the results and discussion section. Your writing will be very interesting if you add an explanation of the appropriate mechanism.
6. Add the lessons learned and limitations of the study.
7. The conclusion should have implications for the future.

Along with this message, we attach the file for the revision. We are looking forward for your revision.

Best regards

Rinto Susilo

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10055	B-ms-review-assignment-1059-Article+Text-9635_12_Desember_2023_revised.docx	December 12, 2023	Article Text
10817	revised_Medical+Sains_Jahe_apoptosis_Flowcyto_3+Feb+2024.docx	February 3, 2024	Article Text
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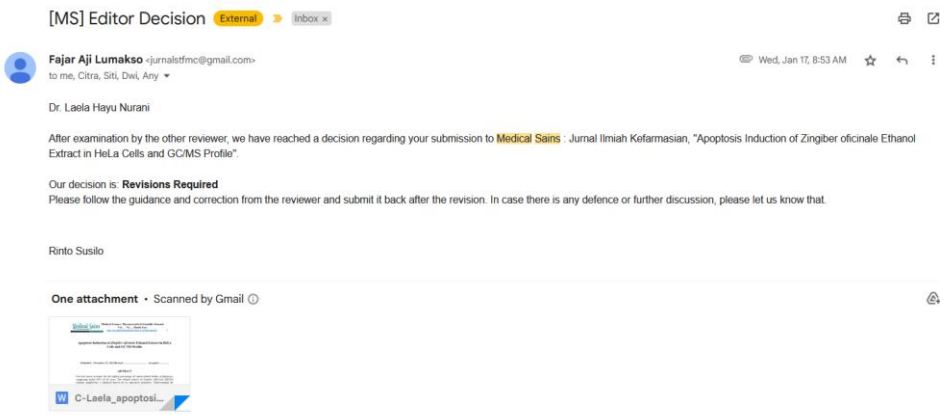
17 Januari 2024



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Fajar Aji Lumakso <jurnalstfmc@gmail.com> Wed, Jan 17, 8:53 AM

to me, Citra, Siti, Dwi, Any

Dr. Laela Hayu Nurani

After examination by the other reviewer, we have reached a decision regarding your submission to Medical Sains : Jurnal Ilmiah Kefarmasian, "Apoptosis Induction of Zingiber oficinale Ethanol Extract in HeLa Cells and GC/MS Profile".

Our decision is: **Revisions Required**

Please follow the guidance and correction from the reviewer and submit it back after the revision. In case there is any defence or further discussion, please let us know that.

Rinto Susilo

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C-Laela_apoptosi...

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3 Feb 2024

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Fajar Aji Lumakso <jurnalstfmc@gmail.com> Sat, Feb 3, 8:48 AM
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Laela Hayu Nurani, Citra Ariani Edityaningrum, Siti Rofida, Dwi Utami, Any Guntarti:

We have reached a decision regarding your submission to Medical Sains : Jurnal Ilmiah Kefarmasian, "Apoptosis Induction of Zingiber oficinale Ethanol Extract in HeLa Cells and GC/MS Profile".

Our decision is to: Accept Submission

Rinto Susilo

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C-revised_Medic...

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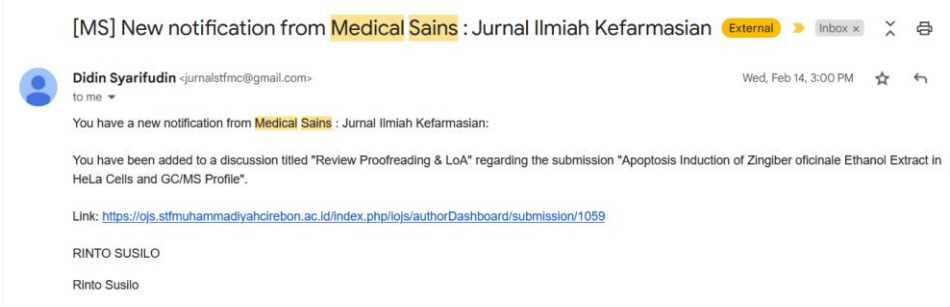
14 Feb:



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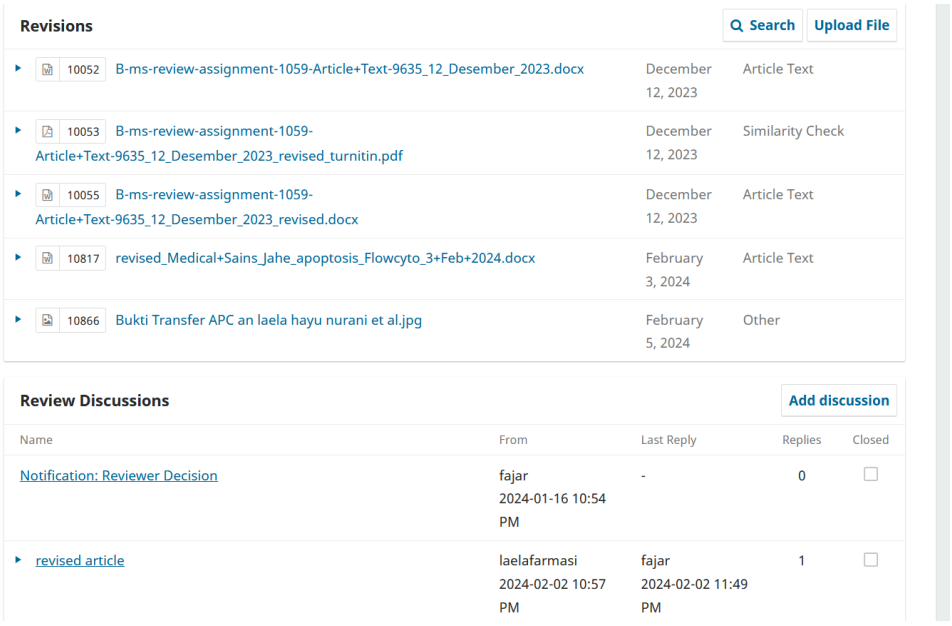
Link: https://ojs.stfmuhammadiyahcirebon.ac.id/index.php/iojs/authorDashboard/submission/1059

RINTO SUSILO

Rinto Susilo



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10817	revised_Medical+Sains_Jahe_apoptosis_Flowcyto_3+Feb+2024.docx	February 3, 2024	Article Text
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Notification: Reviewer Decision	fajar 2024-01-16 10:54 PM	-	0	☐
revised article	laelafarmasi 2024-02-02 10:57 PM	fajar 2024-02-02 11:49 PM	1	☐

Assalamu`alaykum wr wb,

To:
Editor-in-Chief
Journal of Food and Pharmaceutical Science
November 22, 2023
Dear Prof. Dr. Abdul Rohman,
I am pleased to submit the original article entitled “Enhancing Anticancer Potential: Investigating the Synergistic Impact of Doxorubicin and Curcumin on HeLa and Vero Cells in Vitro” for consideration for publication in Journal Food and Pharmaceutical Sciences.

We believe that this manuscript is appropriate for publication by JFPS Journal. The importance of the study for "Enhancing Anticancer Potential: Investigating the Synergistic Impact of Doxorubicin and Curcumin on HeLa and Vero Cells in Vitro " is because it is necessary to reduce the concentration of doxorubicin by curcumin to increase safety in normal cells.

This manuscript has not been published and is not under consideration for publication elsewhere. We have noconflicts of interest to disclose. All authors have approved the manuscript and agree with this submission.

Wassalamu`alaykum wr wb,
Thank you for your
consideration.Sincerely,

Dr. apt. Laela Hayu Nurani, M.Si.
Ahmad Dahlan Halal Center,
Faculty of Pharmacy,
Universitas Ahmad Dahlan,
Yogyakarta, Indonesia
Phone number: +62 898 3464 706/ +62 74 563 515
Fax number: +62 74 564 604

PEMERINTAH PROVINSI JAWA TIMUR
DINAS KESEHATAN
UPT LABORATORIUM HERBAL
MATERIA MEDICA BATU
Jl. Lahor 87 Kota Batu
Jl. Raya 228 Kejayan Kabupaten Pasuruan
Jl. Kolonel Sugiono 457 – 459 Kota Malang
Email : materiamedicabatu@jatimprov.go.id

Nomor : 067/ 1410/ 102.20/ 2023
Sifat : Biasa
Perihal : **Determinasi Tanaman Jahe Merah**

Memenuhi permohonan saudara :

Nama : SITI ROFIDA
NIM : 2136081010
Fakultas : FAKULTAS FARMASI, UNIVERSITAS AHMAD DAHLAN

1. Perihal determinasi tanaman jahe merah
   - Kingdom : Plantae
   - Divisi : Spermatophyta
   - Kelas : Monocotyledonae
   - Bangsa : Zingiberales
   - Suku : Zingiberaceae
   - Marga : Zingiber
   - Jenis : *Zingiber officinale* var. *rubrum* Theilade
   - Nama umum : Jahe merah.
   - Kunci determinasi : 1b-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14a-15a-109a-110b-111b- 112a-113b-116a-119b-120b-128b-129a-130b-132a:Zingiberaceae-1a-2b-6a:Zingiber-1a-2b-6a-7a:*Z.officinale*.
2. Morfologi : Habitus: herba semusim tinggi 40-50 cm. Batang: batang semu, warna hijau beralur dan membentuk rimpang. Daun: tunggal hijau tua, bentuk lanset tepi rata, ujung runcing pangkal tumpul. Bunga: majemuk bentuk bulir sempit dengan ujung runcing, panjang 3-5 cm, lebar 1-2 cm, warna hijau merah, kelopak bunga bentuk tabung bergigi tiga, mahkota bunga ungu, bentuk corong panjang 2-2.5 cm. Buah: bulat panjang warna coklat. Biji: bulat warna hitam. Rimpang: kecil-kecil warna coklat kemerahan. Akar: serabut, putih kotor.
3. Bagian yang digunakan : Rimpang/ Rhizoma.
4. Penggunaan : Penelitian Disertasi.
5. Daftar Pustaka
   - Van Steenis, CGGJ. 2008. *FLORA: untuk Sekolah di Indonesia*. Pradnya Paramita, Jakarta.

Demikian surat keterangan determinasi ini kami buat untuk dipergunakan sebagaimana mestinya.

Batu, 14 Juni 2023

KEPALA UPT LABORATORIUM HERBAL
MATERIA MEDICA BATU

Ir. RATNA YULIANTI, M.M.
Pembina Tk. I
NIP. 19710711 200012 2 002

Induksi Apoptosis Ekstrak Etanol *Zingiber oficinale* pada Sel HeLa dan Profil GC/MS

Laela Hayu Nurani[1*], Siti Rofida[2], Dwi Utami[1], Citra Ariani Edityaningrum[1], dan Any Guntarti[1]

[1] Fakultas Farmasi, Universitas Ahmad Dahlan, Yogyakarta
[2]Fakultas Ilmu Kesehatan, Universitas Muhammadiyah Malang, Malang

**Email Corresponding :* laela.farmasi@pharm.uad.ac.id

Submitted :..... Revised :..... Accepted:.....

ABSTRACT

Kematian karena kanker cerviks menempati posisi tertinggi di Indonesia yaitu mendekati 60% dari keterjadian kanker. Ekstrak etanol *Zingiber officinale* (EEZO) memiliki kandungan kimia zingiberen yang mempunyai aktivitas antikanker. Perlu penelusunan mekanisme induksi apoptosis. Tujuan penelitian ini adalah mengetahui mekanisme apoptosis dan profil GC/MS EEZO. Metode penelitian dimulai dengan melakukan maserasi rimpang ZO dengan etanol 75% sehingga diperoleh EEZO. Uji apoptosis dilakukan pada kelompok kontrol negatif dan kelompok EEZO pada sel HeLa (sel kanker cerviks). Mekanisme apoptosis dilakukan dengan metode fluocytometri FSC-SSC (*Forward scattered light-Side scattered light)*, FTIC (*fluorescein isothiocyanate*), dan PE (*phycoerythrin*). Analisis hasil apoptosis dilakukan dengan membandingkan antara gambaran kontrol dan sampel EEZO yang menunjukkan jumlah sel hidup dan apoptosis serta sel pada fase sub-G1. Identifikasi senyawa dalam EEZO dilakukan dengan GC/MS. Hasil FSC-SSC menunjukkan bahwa kelompok EEZO memiliki sel hidup yang lebih sedikit dibandingkan dengan kontrol. Hasil FTIC menunjukkan bahwa EEZO mampu meningkatkan jumlah kematian sel karena apoptosis dari 68 sel menjadi 1537 sel. Hasil flowcytometer PE menunjukkan bahwa terjadi peningkatan sub-G1 yang menunjukkan adanya induksi apoptosis oleh EEZO. Profil GC/MS menunjukkan bahwa EEZO memiliki 5 komponen yang dominan yaitu: Borneol L, Zingiberene, Farnesol, beta-Sesquiphellandrene, dan Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-. Kesimpulan penelitian ini adalah EEZO mempunyai aktivitas sitotoksik dengan aktivitas apoptosis pada sel HeLa. Terdapat lima senyawa dominan dalam EEZO.

Keywords : apoptosis, HeLa, jahe, flowcytometer

INTRODUCTION

Kanker cervix merupakan kanker dengan kematian yang tertinggi dengan mencapai 60% dari insidensi kanker yang ada. Keterjadian kanker cerviks pada tahun 2018 menduduki urutan kedua setelah kanker payudara dengan insidensi 348.809 dan jumlah kematian 207.210 penderita. Insidensi kanker tiap tahun di Indonesia diperkirakan terdapat 180.000 kasus baru dengan kematian 75% nya (Agustiansyah *et al*., 2021). Penanganan yang sudah dilakukan adalah dengan operasi, radiasi, pengobatan sintesis, maupun pemberian obat herbal. Obat herbal diberikan secara umum pada penderita tumor. Pengembangan obat herbal dilakukan dengan pengujian menggunakan metode *in vitro* dan *in vivo* serta *in silico* dengan penelusuran senyawa aktif dan mekanisme aksi (Rani *et al*., 2023).

Mekanisme antikanker melalui antiproliferasi dan apoptosis dapat menggunakan DNA ladder assay, TUNEL (*Terminal deoxynUcleotidyl transferase Nick-End Labeling*) assay, commet assay (Majtnerová &

Roušar, 2018), flowcytometer, microscopy perfusion platform, genotoxicity specific chromatin changes (Banfalvi, 2017), serta imunositokimia (Jain *et al*., 2019). Penelusuran mekanisme ini dilakukan terutama untuk pengembangan obat herbal dengan kandungan kimia yang beragam. Dengan membandingkan dengan mekanisme kontrol maka akan diperoleh mekanisme definitif senyawa dominan dalam herbal.

Obat herbal yang dikembangkan sebagai obat antikanker banyak berasal dari rimpang / rhizome. Rhizome yang berpotensi sebagai antikanker adalah familia Zingiberaceae. Penelitian yang telah dilakukan atas familia ini adalah *Z. Montanum* (Al-Amin *et al*., 2020), *Z. Zerumbet* (Sithara *et al*., 2018), *Z. Ottensii* (Ruttanapattanakul *et al*., 2021), *Z. Cassumunar* (Han *et al*., 2021), serta *Z. Officinale* (Hasan Mujahid *et al*., 2023). *Z. officinale* mempunyai potensi yang lebih di antara zingiber yang lain sehingga lebih dikembangkan sebagai antikanker (Syafitri *et al*., 2018).

Z. officinale lebih dikembangkan sebagai antikanker karena merupakan tanaman yang dengan metabolit sekunder yang tinggi dan memiliki aktivitas sitotoksik (Nurjannah *et al*., 2022). *Z. officinale* telah diteliti secara in vitro pada HCT116, B16, MCF-7 (Liao *et al*., 2020), sel colon cancer (Mohd Yusof *et al*., 2022), HuH 7 (González-Osuna *et al*., 2023), dan HeLa (Hasan Mujahid *et al*., 2023). Mekanisme aksi yang ditelusur pada pengujian pada sel HeLa adalah sebagai antiproliferasi dengan uji sitotoksi dengan MTT serta mekanisme antioksidan dengan DPPH (Ghazemzadeh *et al*., 2015). Mekanisme antiapoptosis dilakukan dengan pengecatan Hoest (Ansari *et al*., 2016), dan pengecatan Propidium Iode (Hasan Mujahid *et al*., 2023). Namun demikian belum pernah dilakukan analisis induksi apoptosis dengan metode flowcytometri.

Pengujian apoptosis flowcytometer dapat dilakukan dengan pengecatan anexin V, Mekanisme apoptosis dilakukan dengan metode fluocytometri FSC-SSC (*forward scattered light-Side scattered light*), FTIC (*fluorescein isothiocyanate*), dan PE (*phycoerythrin*) (Gadalla *et al*., 2019). Suatu ekstrak dikatakan memiliki aktivitas antikanker dengan metode flowcytometer jika dapat memacu apoptosis yang ditunjukkan oleh jumlah sel mati dan hidup serta jumlah sel yang mengalami apoptosis dan nekrosis dibandingkan dengan kontrol negatif. Selain itu gambaran hasil uji dengan flowcytometer menunjukkan fase sub G1 oleh intervensi sampel menjunjukkan pemacuan apoptosis dibandingkan dengan kontrol negatif (McKinnon, 2018).

Langkah lanjutan sesudah uji aktivitas senyawa adalah identifikasi senyawa yang terdapat di dalam *Z. officinale*. Minyak atsiri merupakan senyawa yang dominan dalam *Z. officinale*. Identifikasi yang dapat dilakukan secara umum dengan menggunakan GC/MS. Analisis GC/MS *Z. officinale* sudah pernah dilakukan (Yu *et al*., 2022). Namun demikian untuk sampel yang diuji juga perlu dilakukan untuk mengetahui profilnya terkait kualitatif dan kuantitatif metabolit sekunder yang dikandung di dalamnya.

Tujuan penelitian ini adalah untuk mengetahui mekanisme apoptosis EEZO pada sel HeLa dengan flowcytometer dan penentuan profil GC/MS.

RESEARCH METHODS

Equipment and Materials

Material yang digunakan adalah Ekstrak etanol Z. Officinale yang diekstraksi dengan etanol teknis 75%, NaOH, Metanol, Aquadest (Bratachem) NaOH (Merck), Metanol p.a (Merck), kultur sel HeLa koleksi Laboratorium Parasitologi FK UGM, aquabidest, natrium bikarbonat (Sigma), hepes (Sigma), Media RPMI, fetal bovine serum (FBS) 10% v/v (Gibco), penisilin-streptomisin 1% v/v (Gibco), fungision0,5% (Gibco), Phosphat buffer saline (PBS) 20% (Sigma), Dimetil sulfoksi (DMSO), Reagen MTT (3-(4,5-Dimethiltiazol-2-il)-2,5-difeniltetrazolium bromide) 5 mg dalam 1 mL PBS (Sigma), Stopper, Sodiun Dodecyl Sulphate SDS) 10% dalam HCL 0,01 N (Merck), 96 well plate, trytan blue stain 0,25% (Gibco). Alat yang digunakan adalah Inkubator CO_2 (New Bronswick, Galaxy 170R) Sentrifuge (Hermle Siemensstr-25 D-78564), Laminar Air Flow Cabinet (Mascotte LH-S, mikropipet (Soccorex), Autoclaf,

hemositometer, ELISA reader (Robonik), Mikroskop inverted (Olympus CKX41-2), GC-MS. EEZO diperoleh dari ekstraksi menggunakan metode maserasi dan pelarut etanol 75% pada rimpang *Z. officinale* yang telah dideterminasi dari UPT Laboratorium Herbal Materia Medica Batu, Dinas Kesehatan Propinsi Jawa Timur dengan nomer 067/1410/102.20/2023.

Research Procedure

1. Uji flowcytometer

Uji flowcytometer dilakukan terhadap sel HeLa yang sudah dikultur dalam 96-well plates dengan kerapatan sel $5x10^4$ sel/sumuran selanjutnya diinkubasi suhu 37°C pada inkubator CO_2 selama 24 jam. Larutan sampel uji (EEZO) ditambahkan pada kultur sel dan diinkubasi pada inkubator CO_2 selama 24 jam. Berikutnya dilakukan pembacaan pada flowcytometer dengan setting output FSC-SSC, FTIC, dan PE di Laboratorium Farmakologi di FKKMK UGM.

2. Uji GC/MS

Pengujian GC-MS dilakukan dengan cara EEZO dilarutkan dalam larutan NaOH dalam Metanol. Sampel sebanyak 1 µL diinjekkan dengan kondisi GC: coloumn oven temperature 70,0°C, Injection temperature 280,00 °C, Injection mode splitless, sampling time 1 minute, Preasure 53,5 kPa, Total flow 21.1 mL/min, coloumn flow 0.91mL/min, Linear velocity 34.9 cm/sec, purge flow 2. mL/min, split ratio 20.0, equilibrium time 3.0 min, MS: Ion source temp 250.00°C, interface temp 160.00°C, Detector gain 1.10kV+0.00kV.

Data Analysis

Data hasil flowcytometer dianalisis dengan membandingkan gambaran hasil flowcytometer antara kontrol dan sampel (EEZO). Proses identifikasi menggunakan alat GC-MS menghasilkan beberapa senyawa-senyawa bioaktif dapat dilihat dari puncak kromatogram sebagai identifikasi. Hasil kromatografi dilanjutkan dengan uji dengan spektrometri massa (MS) dilihat dari spektrum massa dengan masing-masing berat molekul senyawa bioaktif.

RESULTS AND DISCUSSION

Penelitian ini dilakukan atas ethical clearence dari Komite Etik UAD nomor 0123071177. Pengujian induksi apoptosis dilakukan pada sel HeLa oleh pengaruh EEZO dengan flowcytometer dengan melihat gambaran FSC-SSC, FTIC, dan PE.

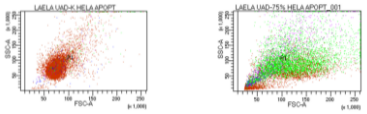
3.1. Pengujian dengan Flowcytometer

3.1.1. FSC-SSC

Instrumen flowcytometer mempunyai sistem optik yang mempunyai kemampuan eksitasi dari laser dan lensa serta optik. Lensa terseut untuk memusatkan sinar laser yang dapat menghasilkan cahaya dengan memindahkan energi elektrok ke orbital yang lebih tinggi yang kemudian dapat jatuh kembali ke orbital yang rendah. Proses ini menghasilkan eksitasi yang terlihat sebagai warna (Adan et al, 2016). Hasil gambaran flowcytometer FSC-SSC sel HeLa yang dipapar dengan EEZO terlihat pada Gambar 1. Sel pada kontrol negatif terlihat tidak menyebar tetapi homogen pada satu tempat yang sama pada posisi yang menunjukkan jumlah sel hidup. Sebaliknya, warna yang dihasilkan pada sel yang dipapar EEZO menunjukkan hamburan yang terlihat posisi yang menunjukkan gambaran sel yang heterogen (Gambar 1). Sel yang apoptosis maupun nekrosis kemungkinan mempengaruhi perbedaan gambaran ini.

Perbedaan gambaran sel pada hasil flowcytometer FSC-SSC karena foton saat menabrak sel akan dibelokkan di sekitar sel sehingga menyebabkan hamburan cahaya yang menjadi dasar gambaran FSC dan SSC terbentuk. FSC sebanding dengan luas permukaan sel, ukuran sel, nukleus, granularitas sel, bentuk sel, serta membran sel. Selanjutnya cahaya SSC sebagian besar dibiaskan, dipantulkan, dan dikumpulkan yang sebanding dengan granularitas sel atau kompleksitas inernal yang sebanding dengan tipe sel dalam populasi yang heterogen (Adan et al, 2016). Sel oleh pengaruh EEZO terlihat heterogen yang dimungkinkan adanya

sel hidup, sel apoptosis, dan nekrosis. Sementara itu, hasil gambaran pada kontrol terlihat sel pada posisi yang homogen sebagai sel yang hidup (Gambar 1).



(a) (b)

Gambar 1. Gambaran sel hasil flowcytometer FSC-SSC sel HeLa yang dipapar dengan ekstrak etanol *Zingiber officinale*

3.1.2. FTIC

Gambaran flowcytometer FTIC memberikan hasil gambaran sel di keempat kuadran. Kuadran kanan bawah menunjukkan persentase sel apoptosis awal, kiri bawah menunjukkan persentase sel yang hidup, kanan atas menunjukkan persentase sel -sel apoptosis akhir, kiri atas menunjukkan persentase sel nekrotik (Mamat et al, 2021). Hasil sel HeLa yang dipapar dengan EEZO terlihat pada Gambar 1 menunjukkan bahwa gambaran flowcytometer FTIC sel HeLa pada kontrol dibandingkan dengan paparan EEZO terdapat perbedaan khususnya pada kuadran kanan atas dan kanan bawah (Gambar 2). Kuadran kanan menunjukkan sel mengalami apoptosis. Disamping itu, EEZO juga meningkatkan kematian sel dengan apoptosis ditunjukkan pada jumlah sel pada kiri atas dibandingkan pada kontrol. EEZO mampu meningkatkan kematian sel ditunjukkan pada kuadran kiri bawah jumlah sel hidup menurun dari 9718 sel menjadi 4159 sel (Tabel 1). Proses kematian sel ini dengan mekanisme apoptosis dan nekrosis.

(a) (b)

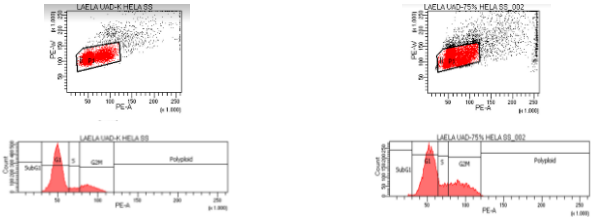
Gambar 2. Gambaran sel hasil flowcytometer FTIC sel HeLa yang dipapar dengan ekstrak etanol *Zingiber officinale*

Table 1. Tabel Jumlah sel dalam setiap kuadran hasil flowcytometer FITC

Kuadran	Interpretasi	Kontrol	EEZO
Kiri bawah	Sel hidup	9718	4159
Kanan atas	Apoptosis awal	109	156
Kanan bawah	Apoptosis akhir	68	1537
Kiri atas	Nekrosis	122	4426

3.1.3. PE

Gambaran flowcytometer PE menghasilkan gambaran jumlah sel yang masuk dalam fase Sub G1 dimana fase ini menunjukkan sel dalam fase istirahat. Fase sub G1 juga dapat menunjukkan sel mengalami proses apoptosis awal (Sheddi et al, 2015). Gambar 3 menunjukkan bahwa fase Sub G1 (bagian kuadran paling kiri dari gambar) sel HeLa meningkat oleh pengaruh dari EEZO. Peningkatan ini sejalan dengan hasil FSC-SSC dan FTIC yang mendukung terjadinya apoptosis oleh pengaruh EEZO. Hasil ini dikuatkan pada Tabel 2 yang menunjukkan penurunan G1 (awal pembelahan sel) dari 6.799 menjadi 4619 serta peningkatan sub G1 dari 29 menjadi 68.



(a) (b)

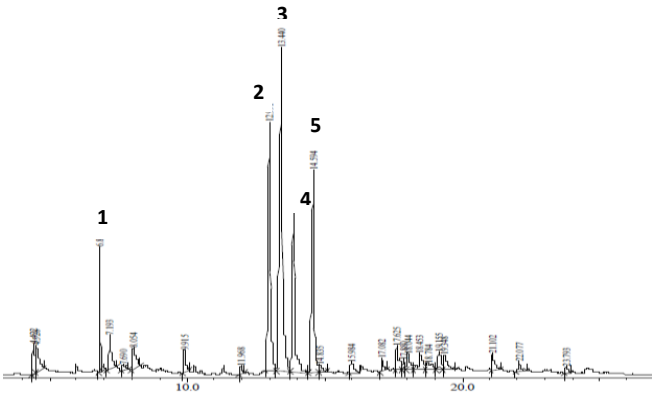
Gambar 3. Hasil FSC-SSC, FITC, dan PE dari (a) Kontrol negatif dan (b) Ekstrak Etanol *Zingiber officinale*

Tabel 2. Jumlah sel dari gambaran flowcytometer PE sel HeLa oleh pengaruh Ekstrak etanol *Zingiber officinale*

Fase sel	Jumlah Sel	
	Kontrol	EEZO
P1	9269	7872
G1	6799	4619
Sub G1	29	68

3.2. Pengujian dengan GC/MS

Profil kromatogram dan identitas senyawa metabolit pada ekstrak etanol 75% rimpang jahe merah hasil analisis dengan menggunakan Kromatografi Gas disajikan pada Gambar 4 dan Tabel 3. Hasil analisis menunjukkan terdapat senyawa metabolit yang termasuk dalam golongan senyawa terpenoida (monoterpen dan sesquiterpen), keton, aldehid, lipida, dan prenol. Profil senyawa metabolit merupakan salah satu indikator yang dapat digunakan untuk memberikan jaminan mutu terhadap aktivitas dan keamanan EEZO sebagai bahan baku produk bahan alam (Balekundri & Mannur, 2020) (Mattoli *et al*., 2023).



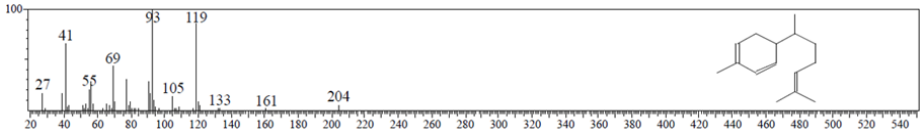
Gambar 4. Profil kromatogram senyawa metabolit ekstrak etanol *Zingiber officinale*

Data kromatogram menunjukkan terdapat puncak tertinggi pada waktu retensi 13,440 menit dengan luas area sebesar 25,16% dari total area seluruh senyawa yang terdeteksi. Data tersebut menunjukkan kelimpahan senyawa tersebut dalam EEZO Senyawa pada waktu retensi tersebut teridentifikasi dengan nama Zingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene) termasuk

golongan senyawa sesquiterpen seperti terlihat pada Tabel 3. Gambar kromatogram MS senyawa Zingiberene disajikan pada Gambar 5.

Tabel 3. Identitas senyawa metabolit dominan hasil analisis dengan menggunakan kromatografi gas

Nomer puncak	Waktu retensi (menit)	Bobot Molekul (m/z)	Rumus Molekul	Identitas Senyawa	Golonga Senyawa (ChemIDplus)	Indeks Kemiripan
1	6.837	154	$C_{10}H_{18}O$	Borneol L	Monoterpenoid	98
2	12.991	202	$C_{15}H_{22}$	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
3	13.440	204	$C_{15}H_{24}$	Zingiberene	Monocyclic Sesquiterpenes	94
4	13.866	222	$C_{15}H_{26}O$	Farnesol	Prenols	93
5	14.594	204	$C_{15}H_{24}$	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94



Gambar 5. Kromatogram MS senyawa hasil GC nomer puncak 3 yang identik dengan zingiberene

Induksi apoptosis pada sel HeLa oleh EEZO dimungkinkan oleh kandungan aktif Zingiberen. Hal ini sejalan dengan penelitian yang dilakukan pada tikus yang diinduksi DMBA (Dimetil benzo antrasen), bahwa zingiberen dapat berperan sebagai kemopreventif (Seshadri, 2022). Selain itu Zingiberene dapat menghambat pertumbuhan sel kanker kolon secara in vitro dan in vivo dengan mekanisme induksi autofagi (Chen et al, 2019).

CONCLUSION

EEZO dapat menginduksi apoptosis dari hasil flowcytometer atas gambaran hasil pada FSC-SSC, FTIC, dan PE. EEZO mengandung komponen utama hasil GC/MS yaitu Borneol L, Zingiberene, Farnesol, beta-Sesquiphellandrene, dan Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-.

ACKNOWLEDGMENT

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REVIEW ARTICLE
Flow cytometry: basic principles and applications

Aysun Adan1*, Gu¨ nel Alizada 2*, Yag˘ mur Kiraz 1,2*, Yusuf Baran 1,2 , and Ayten Nalbant 2	
Journal of Applied Pharmaceutical Science Vol. 11(03), pp 092-099 March, 2021 **Available online at http://www.japsonline.com** **DOI: 10.7324/JAPS.2021.110310** **ISSN 2231-3354Combination effect of cisplatin and gallic acid on apoptosis and antioxidant enzymes level in cervical cancer (HeLa) cells** **Norlida Mamat1,2, Hasmah Abdullah2*, Hermizi Hapidin2, Noor Fatmawati Mokht**	
Novel All Trans-Retinoic Acid Derivatives: Cytotoxicity, Inhibition of Cell Cycle Progression and Induction of Apoptosis in Human Cancer Cell Lines by Ebtesam Saad Al- Sheddi [1], *Molecules* **2015**, *20*(5), 8181-8197; https://doi.org/10.3390/molecules20058181 *Molecules* **2015**, *20*(5), 8181-8197; https://doi.org/10.3390/molecules20058181	
RESEARCH ARTICLE **Zingiberene exerts chemopreventive activity against 7,12-dimethylbenz(a)anthracene-induced breast cancer in Sprague-Dawley rats** Vidya Devanathadesikan Seshadri, Atif Abdulwahab A. Oyouni, Waleed M. Bawazir, Suliman A. Alsagaby, Khalaf F. Alsharif, Ashraf Albrakati, Osama M. Al-Amer **2022**	**https://onlinelibrary.wiley.com/doi/abs/10.1002/jbt.23146**

JBUON 2019; 24(4): **Accepted: 19/01/2019** **Zingiberene inhibits in vitro and in vivo human colon cancer cell growth via autophagy induction, suppression of PI3K/ AKT/mTOR Pathway and caspase 2 deactivation** Hai Chen, Xiaocheng Tang, Ting Liu, Liang Jing, Junhui W	

Apoptosis Induction of *Zingiber oficinale* Ethanol Extract in HeLa Cells and GC/MS Profile

Submitted :November 15, 2023 Revised :..... Accepted:.....

ABSTRACT

Cervical cancer accounts for the highest percentage of cancer-related deaths in Indonesia, comprising nearly 60% of all cases. The ethanol extract of *Zingiber officinale* (EEZO) contains zingiberene, a chemical known for its anticancer properties. Understanding the mechanism behind its apoptosis-inducing effects is crucial. This study aimed to elucidate the apoptotic pathway and analyze the GC/MS profile of EEZO. The research commenced with the maceration of *Zingiber officinale* rhizomes using 75% ethanol to obtain EEZO. Apoptosis assays were conducted on both a negative control group and an EEZO-treated group using HeLa cells (cervical cancer cells). The apoptosis mechanism was evaluated using FSC-SSC (forward scattered light-side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) flow cytometry. Apoptotic results were analyzed by comparing control and EEZO samples, which revealed the number of live cells, apoptotic cells, and cells in the sub-G1 phase. Compound identification in EEZO was performed using GC/MS. FSC-SSC results indicated a lower number of live cells in the EEZO-exposed group compared to the control. FTIC results demonstrated that EEZO significantly increased apoptotic cell death, rising from 68 cells to 1537 cells. PE flow cytometry revealed an elevated sub-G1 population, indicating an induction of apoptosis by EEZO. The GC/MS analysis revealed five dominant components in EEZO: Borneol L, Zingiberene, Farnesol, beta-Sesquiphellandrene, and Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-. In conclusion, this study establishes EEZO's cytotoxic activity through an apoptotic mechanism in HeLa cells, and identifies five major compounds present in EEZO.

Keywords: apoptosis, HeLa, ginger, flow cytometer

INTRODUCTION

Cervical cancer has the highest mortality rate among cancers, accounting for 60% of all reported cases. In 2018, the incidence of cervical cancer ranked second only to breast cancer, with 348,809 reported cases and 207,210 casualties. Indonesia sees an estimated 180,000 new cancer cases annually, with a staggering 75% mortality rate (Agustiansyah et al., 2021). Various treatments have been implemented, including surgery, radiation, synthetic therapies, and the use of herbal medicine, which is typically administered to individuals with tumors. The advancement of herbal remedies involves rigorous testing through in vitro, in vivo, and in silico methods to scrutinize active compounds and their respective mechanisms of action (Rani et al., 2023).

The anticancer mechanisms, targeting antiproliferation and apoptosis, can be assessed through techniques such as the DNA ladder assay, TUNEL (Terminal deoxynucleotidyl transferase Nick-End Labeling) assay, and comet assay, (Majtnerová & Roušar, 2018), flow cytometer, microscopy perfusion platform, genotoxicity specific chromatin changes (Banfalvi, 2017), as well as immunocytochemistry (Jain et al., 2019). The search for this mechanism was carried out mainly for the development of herbal medicines with various chemical contents. By comparing with the control group, a definitive mechanism for the dominant compounds in herbal medicines will be obtained.

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Many herbal medicines developed as anticancer drugs come from rhizomes or rhizomes. Rhizomes that have anticancer potential are in the Zingiberaceae family. Research that has been carried out on this family is *Z. montanum* (Al-Amin et al., 2020), *Z. zerumbet* (Sithara et al., 2018), *Z. ottensii* (Ruttanapattanakul et al., 2021), *Z. cassumunar* (Han et al., 2021), as well as *Z. officinale* (Hasan Mujahid et al., 2023). *Z. officinale* (ZO) has more potential than other zingiber so it is more developed as an anticancer (Syafitri et al., 2018).

Z. officinale is more developed as an anticancer because ZO is a plant with high secondary metabolites and has cytotoxic activity (Nurjannah et al., 2022). *Z. officinale* has been studied in vitro on HCT116, B16, MCF-7(Liao et al., 2020), colon cancer cells (Mohd Yusof et al., 2022), HuH 7 (González-Osuna et al., 2023), and HeLa (Hasan Mujahid et al., 2023) cells. The mechanism of action explored in tests on HeLa cells is anti-proliferation using a cytotoxic test with MTT and an antioxidant mechanism with DPPH (Ghazemzadeh et al., 2015). The antiapoptotic mechanism is carried out by Hoest staining (Ansari et al., 2016), and Propidium Iode painting (Hasan Mujahid et al., 2023). However, analysis of apoptosis induction has never been carried out using the flow cytometry method.

Apoptosis testing with a flow cytometer can be done by staining with anexin V. The mechanism of apoptosis can be observed from the test results using the FSC-SSC (forward scattered light-Side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) fluocy tometry methods (Gadalla et al., 2019). An extract is said to have anticancer activity using the flow cytometry method if it can stimulate apoptosis as indicated by the number of dead and live cells as well as the number of cells experiencing apoptosis and necrosis compared with the negative control. Apart from that, the test results with a flow cytometer showed that the sub-G1 phase by sample intervention showed accelerated apoptosis compared to the negative control (McKinnon, 2018).

The next step after testing compound activity is identifying the compounds contained in *Z. officinale*. Essential oils are the dominant compounds in *Z. officinale*. Identification can be done generally using GC/MS. GC/MS analysis of *Z. officinale* has been carried out (Yu et al., 2022). However, for the samples being tested, it is also necessary to determine the profile regarding the qualitative and quantitative secondary metabolites contained there in. Therefore, the aim of this study was to determine the mechanism of EEZO apoptosis in HeLa cells using a flow cytometer and determine the GC/MS profile.

RESEARCH METHODS

Equipment and Materials

The material used is *Z. officinale* ethanol extract which is extracted with 75% technical ethanol, NaOH, methanol, Aquadest aquadest (Bratachem) NaOH (Merck), Methanol pa (Merck), HeLa cell culture collection from the Faculty of Medicine UGM Parasitology Laboratory, aquabidest, sodium bicarbonate (Sigma), hepes (Sigma), RPMI media, fetal bovine serum (FBS) 10% v/ v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungision 0.5% (Gibco), pPhosphate buffer saline (PBS) 20% (Sigma), dDimethyl sulfoxy (DMSO), MTT Reagent (3-(4 ,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg in 1 mL PBS (Sigma), sStopper, sSodiun dDodecyl sSulphate SDS) 10% in 0.01 N HClL (Merck), 96 well plate, trytan blue stain 0.25% (Gibco). The equipment used is a CO_2 iIncubator (New Bronswick, Galaxy 170R), cCentrifuge (Hermle Siemensstr-25 D-78564), lLaminar aAir fFlow cCabinet (Mascotte LH-S, micropipette (Soccorex), aAutoclave, hemocytometer, ELISA microplate reader (Robonik), MMicroscope inverted (Olympus CKX41-2), GC-MS. EEZO was obtained from extraction using the maceration method and 75% ethanol solvent on *Z. officinale* rhizomes which had been determined from UPT Batu Herbal Materia Medica Laboratory, East Java Provincial Health Service with number 067/1410/102.20/2023.

Research Procedures

3. Flow cytometer test

The flow cytometer test was carried out on HeLa cells that had been cultured in depth 96-well plates with a cell density of $5x10^4$ cells/well and were then incubated at 37°C in a CO_2 incubator for 24 hours.

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The test sample solution (EEZO) was added to the cell culture and incubated in a CO_2 incubator for 24 hours. Next, readings are taken on the flow cytometer with the output settings FSC-SSC, FTIC, and PE in the Pharmacology Laboratory at FKKMK UGM.

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4. GC/MS test

GC-MS testing was carried out by dissolving EEZO in a NaOH solution in methanol. A sample of 1 µL was injected with GC conditions: column oven temperature 70.0 °C, injection temperature 280.00 °C, injection mode splitless, sampling time 1 minute, pressure 53.5 kPa, total flow 21.1 mL/min, column flow 0.91 mL/min, linear velocity 34.9 cm/sec, purge flow 2. mL/min, split ratio 20.0, equilibrium time 3.0 min, MS: ion source temp 250.00 °C, interface temp 160.00 °C, detector gain 1.10kV+0.00 kV.

Data Analysis

Flow cytometer results data were analyzed by comparing the flow cytometer results between the control and sample (EEZO). The identification process using the GC-MS tool produces several bioactive compounds that can be seen from the chromatogram peaks for identification. The chromatography results were followed by testing with mass spectrometry (MS) seen from the mass spectrum with each molecular weight of the bioactive compound.

RESULTS AND DISCUSSION

This research was conducted based on ethical clearance from the UAD Ethics Committee number 0123071177. Apoptosis induction testing was carried out on HeLa cells by the influence of EEZO with a flow cytometer by looking at the FSC-SSC, FTIC, and PE images.

3.1. Testing with a Flowcytometer

3.1.1. FSC-SSC

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The flow cytometer instrument has an optical system that has excitation capabilities from lasers and lenses as well as optics. The lens is used to focus laser beams which can produce light by moving electrical energy to higher orbitals which can then fall back to lower orbitals. This process produces excitation that is visible as color (Adan et al., 2016). The results of the FSC-SSC flow cytometer image of HeLa cells exposed to EEZO are shown in Figure 1. The cells in the negative control do not appear to be spread out but are homogeneous in the same place at a position that shows the number of live cells. In contrast, the color produced in cells exposed to EEZO showed visible scattering of heterogeneous cell image positions (Figure 1). Apoptotic or necrotic cells may influence these differences in appearance.

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Why you check the apoptosis mechanism?
Explain the apoptosis in cells

The difference in cell images in the FSC-SSC flow cytometer results is because photons when they hit the cell will be deflected around the cell, causing light scattering ~~on the basis of~~ based on which the FSC and SSC images are formed. FSC is proportional to cell surface area, cell size, nucleus, cell granularity, cell shape, and cell membrane. Furthermore, SSC light is mostly refracted, reflected, and collected which is proportional to the cell granularity or internal complexity which is proportional to the cell type in a heterogeneous population (Adan et al., 2016). Cells affected by EEZO appear heterogeneous, which is possible by the presence of live cells, apoptotic cells, and necrosis cells. Meanwhile, the control images show cells in a homogeneous position as the dominant living cells (Figure 1).

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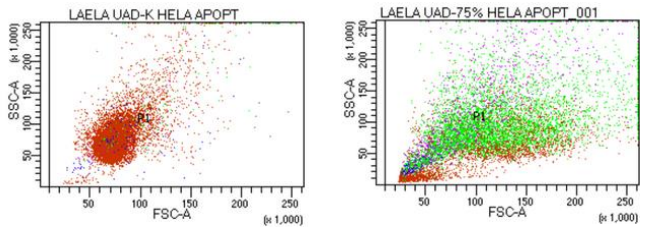
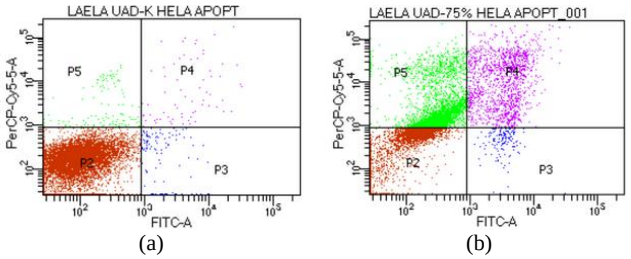


(a) (b)

Figure 1. Image of cells from the FSC-SSC flow cytometer of HeLa cells exposed to *Zingiber officinale* ethanol extract (a). control; (b). EEZO

3.1.2. FTIC

The results of the FTIC flow cytometer provide an overview of cells in all four quadrants. The lower right quadrant shows the percentage of early apoptotic cells, the lower left shows the percentage of viable cells, and the upper right shows the percentage of late apoptotic cells, the upper left shows the percentage of necrotic cells (Mamat et al., 2021).~~The results of HeLa cells exposed to EEZO shown in Figure 2 show that~~ the FTIC ~~flow cytometer~~ results of HeLa cells in controls compared to those exposed to EEZO have differences, especially in the upper right and lower right quadrants (Figure 2). The right quadrant shows cells undergoing apoptosis. Besides that, EEZO also increased cell death with apoptosis as indicated by the increase in the number of cells seen in the upper left quadrant compared to the control. EEZO was also able to increase cell death as shown in the lower left quadrant, the number of living cells decreased from 9718 cells to 4159 cells (Table 1). This cell death process is by apoptosis and necrosis mechanisms.



(a) (b)

Figure 2. Image of cells from the FTIC flow cytometer of HeLa cells exposed to Zingiber officinale ethanol extract (a). control, (b) EEZO

Table 1. Table: Number of cells in each quadrant of FITC flow cytometer results by the influence of *Zingiber officinale* ethanol extract

Quadrant	Interpretation	Control	EEZO
Bottom left	Living cells	9718	4159
Top right	Early apoptosis	109	156
Bottom right	Final apoptosis	68	1537
Top left	Necrosis	122	4426

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3.1.3. P.E

The PE flow cytometer image produces an image of the number of cells entering the ~~S~~sub-G1 phase, where this phase shows cells in the resting phase. The sub-G1 phase can also show cells undergoing the initial apoptosis process (Al-Sheddi et al., 2015). Figure 3 shows that the ~~S~~sub-G1 phase (leftmost quadrant of the figure) of HeLa cells is increased by the influence of EEZO. This increase is in line with the results of FSC-SSC and FTIC which support the occurrence of apoptosis due to the influence of EEZO. This result is confirmed in Table 2 which shows a decrease in G1 (beginning of cell division) from 6,799 to 4619 and an increase in sub G1 from 29 to 68.

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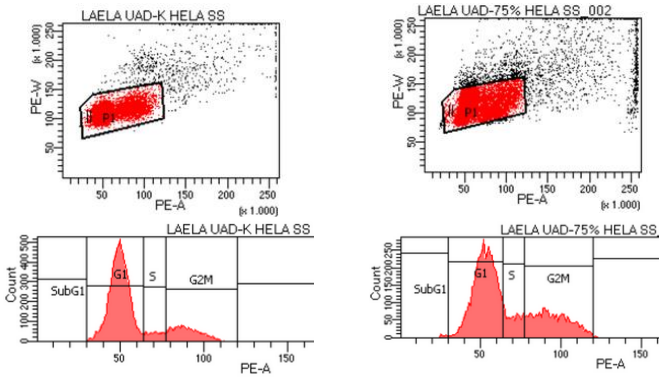

(a) (b)

Figure 3. FSC-SSC, FITC, and PE results from (a) Negative control and (b) Ethanol Extract of *Zingiber officinale*

Table 2. Number of cells from PE flow cytometer images of HeLa cells by the influence of *Zingiber officinale* ethanol extract

Cell phase	Number of Cells	
	Control	EEZO
P1	9269	7872
G1	6799	4619
Sub G1	29	68

3.2. Testing with GC/MS

The chromatogram profile and identity of metabolite compounds in the 75% ethanol extract of red ginger rhizomes, the results of analysis using ~~G~~gas c~~C~~hromatography (GC), are presented in Figure 4 and Table 3. The results of the analysis show that there are metabolite compounds ~~which are~~that included in the group of terpenoid compounds (monoterpenes and sesquiterpenes), ketones, aldehydes, lipids, and prenols. The metabolite compound profile is an indicator that can be used to provide quality assurance regarding the activity and safety of EEZO as a raw material for natural products (Balekundri & Mannur, 2020; Mattoli et al., 2023).

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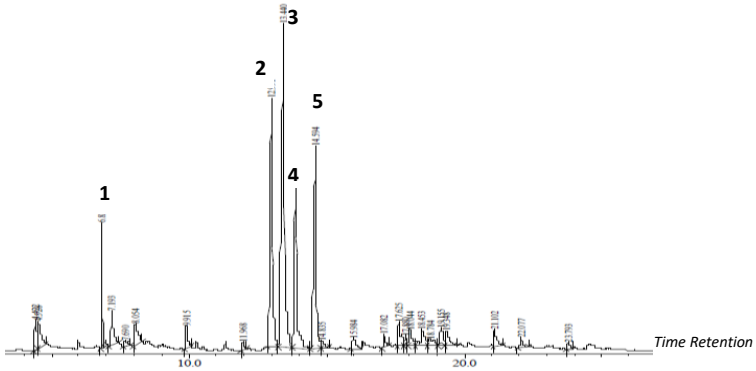

Figure 4. Chromatogram profile of metabolite compounds from *Zingiber officinale* ethanol extract

The chromatogram data in Figure 4 shows that there is the highest peak at a retention time of 13,440 minutes with an area of 25.16% of the total area of all detected compounds. These data show the abundance of these compounds in EEZO. The compounds at the retention time are identified by name Zingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene) belongs to the sesquiterpene group of compounds as shown in Table 3. Results This is in line with the MS results showing the zingiberene compound in Figure 5.

Table 3. Identity of the dominant metabolite compounds of *Zingiber officinale* ethanol extract from analysis using gas chromatography

Peak number	Retention time (minute)	Molecular Weight (m/z)	Molecular Formula	Compound Identity	Compound Classes (ChemIDplus)	Similarity Index
1	6,837	154	C10H18O	Borneol L	Monoterpenoids	98
2	12,991	202	C15H22	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
3	13,440	204	C15H24	Zingiberene	Monocyclic Sesquiterpenes	94
4	13,866	222	C15H26O	Farnesol	Prenols	93
5	14,594	204	C15H24	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94

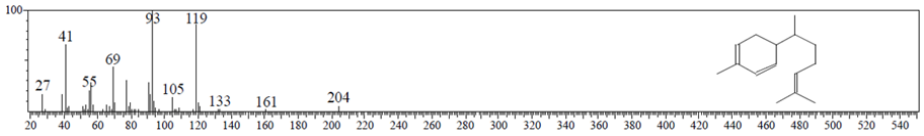

Figure 5. Image of molecular fragmentation on MS of the compound resulting from GC peak number 3 which is identical to zingiberene

Induction of apoptosis in HeLa cells by EEZO is made possible by the active ingredient Zingiberen. This is in line with research conducted on rats induced by DMBA (Dimethyl benzo anthracene), that

Commented [AR21]: Provide possible mechanisms of apoptosis from zingiberene or from previous studies

zingiberene can act as a chemopreventive (Seshadri, 2022). In addition, Zingiberene can inhibit the growth of colon cancer cells in vitro and in vivo by inducing autophagy (Chen et al., 2019).

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CONCLUSION

EEZO can induce apoptosis from the flow cytometer results aboveFSC-SSC, FTIC, and PE. EEZO contains the main components of GC/MS results, namely Borneol L,Zingiberene, Farnesol, beta-Sesquiphellandrene, and Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-.

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ACKNOWLEDGMENT

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Apoptosis Induction of *Zingiber oficinale* Ethanol Extract in HeLa Cells and GC/MS Profile

Submitted :November 15, 2023 Revised :..... Accepted:.....

ABSTRACT

Cervical cancer accounts for the highest percentage of cancer-related deaths in Indonesia, comprising nearly 60% of all cases. The ethanol extract of *Zingiber officinale* (EEZO) contains zingiberene, a chemical known for its anticancer properties. Understanding the mechanism behind its apoptosis-inducing effects is crucial. This study aimed to elucidate the apoptotic pathway and analyze the GC/MS profile of EEZO. The research commenced with the maceration of *Zingiber officinale* rhizomes using 75% ethanol to obtain EEZO. Apoptosis assays were conducted on both a negative control group and an EEZO-treated group using HeLa cells (cervical cancer cells). The apoptosis mechanism was evaluated using FSC-SSC (forward scattered light-side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) flow cytometry. Apoptotic results were analyzed by comparing control and EEZO samples, which revealed the number of live cells, apoptotic cells, and cells in the sub-G1 phase. The dominant components in the EEZO that had potential as apoptosis inductors were detected using GC/MS FSC-SSC results indicated a lower number of live cells in the EEZO-exposed group compared to the control. FTIC results demonstrated that EEZO significantly increased apoptotic cell death, rising from 68 cells to 1537 cells. PE flow cytometry revealed an elevated sub-G1 population, indicating an induction of apoptosis by EEZO. The GC/MS analysis revealed five dominant components in EEZO which had potential as apoptosis inductors were: Borneol L, Zingiberene, Farnesol, beta-Sesquiphellandrene, and Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-. In conclusion, EEZO with its dominant compound, Zingeberen, induces apoptosis in HeLa cells and, therefore, indicates anticancer potentials.

Keywords: cancer, ginger, flow cytometry, HeLa

INTRODUCTION

Cervical cancer has the highest mortality rate among cancers, accounting for 60% of all reported cases. In 2018, the incidence of cervical cancer ranked second only to breast cancer, with 348,809 reported cases and 207,210 casualties. Indonesia sees an estimated 180,000 new cancer cases annually, with a staggering 75% mortality rate (Agustiansyah et al., 2021). Various treatments have been implemented, including surgery, radiation, synthetic therapies, and the use of herbal medicine, which is typically administered to individuals with tumors (Mosaddad et al., 2021). The advancement of herbal remedies involves rigorous testing through *in vitro*, *in vivo*, and *in silico* methods to scrutinize active compounds and their respective mechanisms of action (Rani et al., 2023).

The anticancer mechanisms, targeting antiproliferation and apoptosis, can be assessed through techniques such as the DNA ladder assay, TUNEL (Terminal deoxynucleotidyl transferase Nick-End Labeling) assay, and comet assay, (Majtnerová & Roušar, 2018), flow cytometer, microscopy perfusion platform, genotoxicity specific chromatin changes (Banfalvi, 2017), as well as immunocytochemistry (Jain et al., 2019). The search for this mechanism was carried out mainly for the development of herbal medicines with various chemical contents.

Many herbal medicines developed as anticancer drugs come from leaves, flower, root and rhizomes (Khan et al., 2020). Rhizomes that have anticancer potential are in the Zingiberaceae family. Research that

has been carried out on this family is *Z. montanum* (Al-Amin et al., 2020), *Z. zerumbet* (Sithara et al., 2018), *Z. ottensii* (Ruttanapattanakul et al., 2021), *Z. cassumunar* (Han et al., 2021), as well as *Z. officinale* (Hasan Mujahid et al., 2023). *Z. officinale* (ZO) has more potential than other zingiber so it is more developed as an anticancer (Syafitri et al., 2018).

Z. officinale is more developed as an anticancer because ZO is a plant with high secondary metabolites and has cytotoxic activity (Nurjannah et al., 2022). *Z. officinale* has been studied *in vitro* on HCT116, B16, MCF-7 (Liao et al., 2020), colon cancer cells (Mohd Yusof et al., 2022), HuH 7 (González-Osuna et al., 2023), and HeLa cells (Hasan Mujahid et al., 2023). The mechanism of action explored in tests on HeLa cells is anti-proliferation using a cytotoxic test with MTT and an antioxidant mechanism with DPPH (Ghazemzadeh et al., 2015). The antiapoptotic mechanism is carried out by Hoest staining (Ansari et al., 2016), and Propidium Iode painting (Hasan Mujahid et al., 2023). However, from the method that has been conducted, its effect on the cancer cell cycle to the level of the quantity of the cells is unknown. Therefore, assessing the mechanism using the flow cytometry method is required. Flow cytometry results can provide information on the G1 cycle (cell cycle entering the inactive phase). Thus, it is important to utilize a flow cytometer to determine the mechanism of apoptosis orcell death.

An extract is said to have anticancer activity using the flow cytometry method if it can stimulate apoptosis as indicated by the number of dead and live cells as well as the number of cells experiencing apoptosis and necrosis compared with the negative control. Apart from that, the test results with a flow cytometer showed that the sub-G1 phase by sample intervention showed accelerated apoptosis compared to the negative control (McKinnon, 2018). Apoptosis testing with a flow cytometer can be done by staining with anexin V. The mechanism of apoptosis can be observed from the test results using the FSC-SSC (forward scattered light-Side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) fluocy tometry methods (Gadalla et al., 2019).

The next step after testing compound activity is identifying the compounds contained in *Z. officinale* in this regards has anticancer potential with apoptosis mechanism. Essential oils are the dominant compounds in *Z. officinale*. Identification can be done generally using GC/MS. GC/MS analysis of *Z. officinale* has been carried out (Yu et al., 2022). This study aims to determine the mechanism of EEZO in inducing apoptosis using the flow cytometer method and to identify its dominant compound that responsible for this effect.

RESEARCH METHODS

Equipment and Materials

The material used is EEZO which is extracted with 75% technical ethanol, methanol, aquadest (Bratachem), NaOH (Merck), methanol pa (Merck), HeLa cell culture collection from the Parasitology Laboratory Faculty of Medicine Public Health and Nursing UGM, aquabidest, sodium bicarbonate (Sigma), hepes (Sigma), Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), fetal bovine serum (FBS) 10% v/ v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungision 0.5% (Gibco), phosphate buffer saline (PBS) 20% (Sigma), dimethyl sulfoxy (DMSO), MTT Reagent (3-(4 ,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg in 1 mL PBS (Sigma), stopper, sodiun dodecyl sulphate SDS) 10% in 0.01 N HCl (Merck), 96 well-plate, trytan blue stain 0.25% (Gibco). The equipment used is a CO_2 incubator (New Bronswick, Galaxy 170R), centrifuge (Hermle Siemensstr-25 D-78564), laminar air flow cabinet (Mascotte LH-S, micropipette (Soccorex), autoclave, hemocytometer, ELISA microplate reader (Robonik), microscope inverted (Olympus CKX41-2), GC-MS. EEZO was obtained from extraction using the maceration method and 75% ethanol solvent on *Z. officinale* rhizomes which had been determined from UPT Batu Herbal Materia Medica Laboratory, East Java Provincial Health Service with number 067/1410/102.20/2023.

Research Procedures

5. Flow cytometer test

The flow cytometer test was carried out on HeLa cells that had been cultured in-depth 96-well plates with a cell density of $5x10^4$ cells/well and were then incubated at 37°C in a CO_2 incubator for 24 hours. The test sample solution (EEZO) was added to the cell culture and incubated in a CO_2 incubator for 24 hours. Next, readings are taken on the flow cytometer with the output settings FSC-SSC, FTIC, and PE in the Pharmacology Laboratory at Faculty of Medicine Public Health and Nursing UGM.

6. GC/MS test

GC-MS testing was carried out by dissolving EEZO in a NaOH solution in methanol. A sample of 1 µL was injected with GC conditions: column oven temperature 70.0 °C, injection temperature 280.00 °C, injection mode splitless, sampling time 1 minute, pressure 53.5 kPa, total flow 21.1 mL/min, column flow 0.91 mL/min, linear velocity 34.9 cm/sec, purge flow 2. mL/min, split ratio 20.0, equilibrium time 3.0 min, MS: ion source temp 250.00 °C, interface temp 160.00 °C, detector gain 1.10kV+0.00 kV.

Data Analysis

Flow cytometer results data were analyzed by comparing the flow cytometer results between the control and sample (EEZO). The identification process using the GC-MS tool produces several bioactive compounds that can be seen from the chromatogram peaks for identification. The chromatography results were followed by testing with mass spectrometry (MS) seen from the mass spectrum with each molecular weight of the bioactive compound.

RESULTS AND DISCUSSION

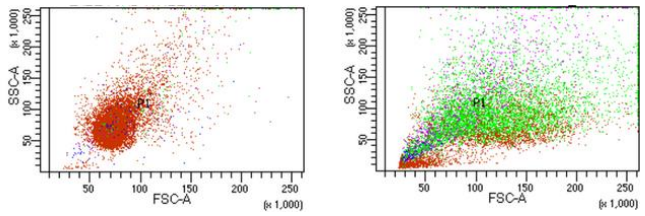
This research was conducted based on ethical clearance from the UAD Ethics Committee number 0123071177. Apoptosis induction testing was carried out on HeLa cells by the influence of EEZO with a flow cytometer by looking at the FSC-SSC, FTIC, and PE images.

3.1. Testing apoptosis with a flowcytometer

3.1.1. *Apoptosis and the flowcytometer FSC-SSC*

Apoptosis can be detected with a flow cytometer based on the number of cells seen as a result of excitation scattering of cells that occurs. This is becouse the scattering produces colors that can be detected in the instrument (Adan et al., 2016). The results of the FSC-SSC flow cytometer image of HeLa cells exposed to EEZO are shown in Figure 1. The cells in the negative control do not appear to be spread out but are homogeneous in the same place at a position that shows the number of live cells. In contrast, the color produced in cells exposed to EEZO showed visible scattering of heterogeneous cell image positions (Figure 1). Apoptotic or necrotic cells may influence these differences in appearance.

The difference in cell images in the FSC-SSC flow cytometer results is because photons when they hit the cell will be deflected around the cell, causing light scattering based on which the FSC and SSC images are formed. FSC is proportional to cell surface area, cell size, nucleus, cell granularity, cell shape, and cell membrane. Furthermore, SSC light is mostly refracted, reflected, and collected which is proportional to the cell granularity or internal complexity which is proportional to the cell type in a heterogeneous population (Adan et al., 2016). The test was carried out at IC_{50} EEZO on HeLa cells so that the cells could be clearly observed in sufficient numbers and was carried out in one test. Cells affected by EEZO appear heterogeneous, which is possible by the presence of live cells, apoptotic cells, and necrosis cells. Meanwhile, the control images show cells in a homogeneous position as the dominant living cells (Figure 1).

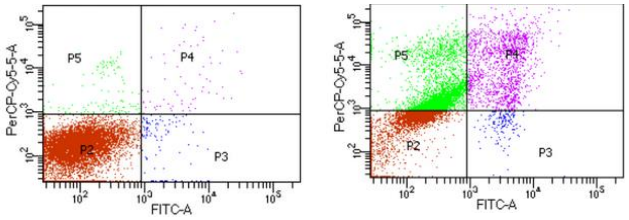


(a) (b)

Figure 1. Image of cells from the FSC-SSC flow cytometer of HeLa cells exposed to *Zingiber officinale* ethanol extract (a). control; (b). EEZO

3.1.2. *Apoptosis and the flowcytometer results FTIC*

The results of the FTIC flow cytometer provide an overview of cells in all four quadrants. The lower right quadrant shows the percentage of early apoptotic cells, the lower left shows the percentage of viable cells, and the upper right shows the percentage of late apoptotic cells, the upper left shows the percentage of necrotic cells (Mamat et al., 2021). The FTIC results of HeLa cells in controls compared to those exposed to EEZO have differences, especially in the upper right and lower right quadrants (Figure 2). The right quadrant shows cells undergoing apoptosis. Besides that, EEZO also increased cell death with apoptosis as indicated by the increase in the number of cells seen in the upper left quadrant compared to the control. EEZO was also able to increase cell death as shown in the lower left quadrant, the number of living cells decreased from 9718 cells to 4159 cells (Table 1). This cell death process is by apoptosis and necrosis mechanisms.



(a) (b)

Figure 2. Image of cells from the FTIC flow cytometer of HeLa cells exposed to *Zingiber officinale* (EEZO) ethanol extract (a). control, (b) EEZO

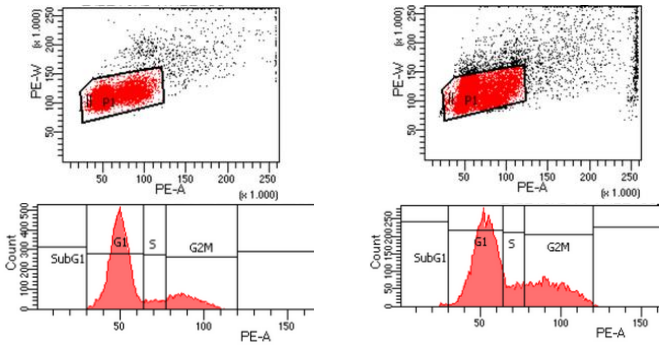
Table 1. Table: Number of cells in each quadrant of FITC flow cytometer results by the influence of *Zingiber officinale* ethanol extract

Quadrant	Interpretation	Control	EEZO
Bottom left	Living cells	9718	4159
Top right	Early apoptosis	109	156
Bottom right	Final apoptosis	68	1537
Top left	Necrosis	122	4426

3.1.3. *Apoptosis and the flowcytometer results P.E*

The cell cycle that can be observed from the flow cytometry results include the number of cells in the cell growth (G1), synthesis (S), arrest (G2/M) phases, apoptosis in the sub cell growth (sub-G1) phase. Apoptosis can be observed from the results of the sub-G1 phase of the cell cycle from the results of cell counts on the flow cytometer between intervention samples compared to controls. The PE flow cytometer

image produces an image of the number of cells entering the sub-G1 phase, where this phase shows cells in the resting phase. The sub-G1 phase can also show cells undergoing the initial apoptosis process (Al-Sheddi et al., 2015). Figure 3 shows that the sub-G1 phase (leftmost quadrant of the figure) of HeLa cells is increased by the influence of EEZO. This increase is in line with the results of FSC-SSC and FTIC which support the occurrence of apoptosis due to the influence of EEZO. This result is confirmed in Table 2 which shows a decrease in G1 (beginning of cell division) from 6,799 to 4619 and an increase in sub-G1 from 29 to 68. The results that support the mechanism by which apoptosis occurs are an increase in the cell resting or antiproliferation phase (G2/M) from 1813 to 2318, although the S phase increases from 626 to 849 as a consequence of increasing G2/M. However, the increased synthesis due to EEZO here does not cause the number of cells to increase because it then enters the G2/M phase. This is also supported by the smaller number of cells compared to the control, namely 15744 by the influence of EEZO from the number 18538 in the control. The limitation of this research is the compound that is responsible for the apoptosis mechanism contained in EEZO. However, identifying the dominant compound can provide important information regarding the content of compounds that may have cytotoxic activity from EEZO.



(a) (b)

Figure 3. FSC-SSC, FITC, and PE results from (a) Negative control and (b) Ethanol Extract of *Zingiber officinale*

Table 2. Number of cells from PE flow cytometer images of HeLa cells by the influence of *Zingiber officinale* ethanol extract

Cell phase	Number of Cells	
	Control	EEZO
P1	9269	7872
G1	6799	4619
S	626	849
G2M	1813	2318
Sub-G1	29	68
Polyploid	2	18
Sum	18538	15744

3.2. Identification of the dominant compound in EEZO as an inductor apoptosis using GC/MS

The chromatogram profile and identity of metabolite compounds in the 75% ethanol extract of red ginger rhizomes, the results of analysis using gas chromatography (GC), are presented in Figure 4 and Table 3. The results of the analysis show that there are metabolite compounds that included in the group of terpenoid compounds (monoterpenes and sesquiterpenes), ketones, aldehydes, lipids, and phenols. The

metabolite compound profile is an indicator that can be used to provide quality assurance regarding the activity and safety of EEZO as a raw material for natural products (Balekundri & Mannur, 2020; Mattoli et al., 2023).

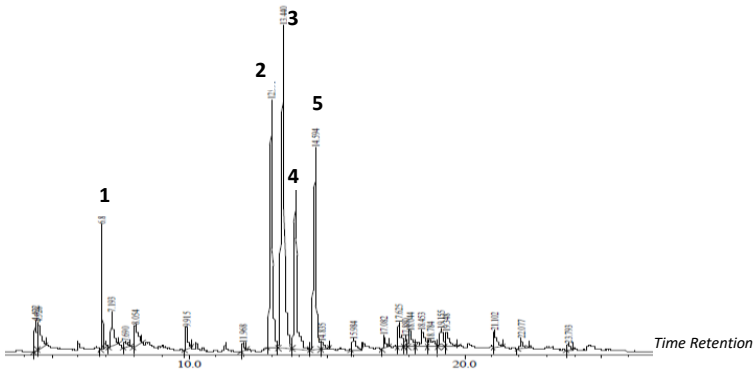


Figure 4. Chromatogram profile of metabolite compounds from *Zingiber officinale* ethanol extract

The chromatogram data in Figure 4 shows that there is the highest peak at a retention time of 13,440 minutes with an area of 25.16% of the total area of all detected compounds. These data show the abundance of these compounds in EEZO. The compounds at the retention time are identified by nameZingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene) belongs to the sesquiterpene group of compounds as shown in Table 3. Results This is in line with the MS results showing the zingiberene compound in Figure 5.

Table 3. Identity of the dominant metabolite compounds of *Zingiber officinale* ethanol extract from analysis using gas chromatography

Peak number	Time retention (minute)	Molecular Weight (m/z)	Molecular Formula	Compound Identity	Compound Classes (ChemIDplus)	Similarity Index
1	6,837	154	$C_{10}H_{18}O$	Borneol L	Monoterpenoids	98
2	12,991	202	$C_{15}H_{22}$	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
3	13,440	204	$C_{15}H_{24}$	Zingiberene	Monocyclic Sesquiterpenes	94
4	13,866	222	$C_{15}H_{26}O$	Farnesol	Prenols	93
5	14,594	204	$C_{15}H_{24}$	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94

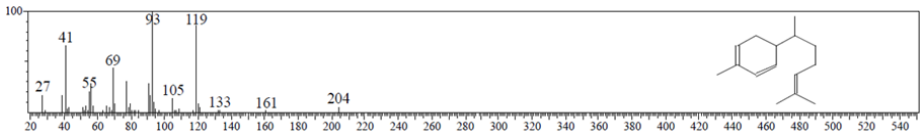


Figure 5. Image of molecular fragmentation on MS of the compound resulting from GC peak number 3 which is identical to zingiberene

Induction of apoptosis in HeLa cells by EEZO is made possible by the active ingredient Zingiberen. Zingiberen can significantly increase the formation of ROS which causes cell apoptosis (Hasan Mujahid et al., 2023) and an increase in sub-G1 in SiHa cells (Lee, 2016). In *vivo* tests were carried out on rats induced by DMBA (Dimethyl benzo anthracene), that zingiberene can act as a chemopreventive (Seshadri, 2022). In addition, Zingiberene can inhibit the growth of colon cancer cells *in vitro* and *in vivo* by inducing autophagy (Chen et al., 2019).

CONCLUSION

EEZO, with its main content the Zingiberene, increases apoptosis in HeLa cells, so it has potential as an anticancer.

ACKNOWLEDGMENT

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Apoptosis Induction of *Zingiber oficinale* Ethanol Extract in HeLa Cells and GC/MS Profile

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The Apoptosis Induction of Zingiber officinale ethanolic extraxt-treated HeLa Human Cervical Cancer Cells and Active Compound Profiling using GC-MS

Submitted : November 15, 2023 Revised :..... Accepted:.....

ABSTRACT

Cervical cancer accounts for the highest percentage of cancer-related deaths in Indonesia, comprising nearly 60% of all cases. The ethanol extract of *Zingiber officinale* (EEZO) contains zingiberene, a chemical known for its anticancer properties. Understanding the mechanism behind its apoptosis-inducing effects is crucial. This study aimed to elucidate the apoptotic pathway and analyze the GC/MS profile of EEZO. The research commenced with the maceration of *Zingiber officinale* rhizomes using 75% ethanol to obtain EEZO. Apoptosis assays were conducted on both a negative control group and an EEZO-treated group using HeLa cells (cervical cancer cells). The apoptosis mechanism was evaluated using FSC-SSC (forward scattered light-side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) flow cytometry. Apoptotic results were analyzed by comparing control and EEZO samples, which revealed the number of live cells, apoptotic cells, and amount of cells in the sub-G1 phase. The dominant components in the EEZO that had potential as apoptosis inductors were detected using GC/MS FSC-SSC results indicated a lower number of viable cells in the EEZO-exposed group compared to the control. FTIC results demonstrated that EEZO significantly increased apoptotic cell death, rising from 68 cells to 1537 cells. PE flow cytometry revealed an elevated sub-G1 population, indicating an induction of apoptosis by EEZO. The GC/MS analysis revealed five dominant components in EEZO which had potential as apoptosis inducers were: L-borneol, zingiberene, farnesol, beta-sesquiphellandrene, and benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl-. In conclusion, EEZO with its dominant compound, Zingeberen, induces apoptosis in HeLa cells and, therefore, indicates anticancer potentials.

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Keywords: cancer, ginger, flow cytometry, HeLa, zingiberene

INTRODUCTION

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2.In case there is no report on how zingiber acts as anticancer, this is your turn to tell the novelty of this esearch
3.Please tell the readers why GC/MS is important because we still found that the GC/MS has not been introduced clearly

Cervical cancer has the highest mortality rate among cancers, accounting for 60% of all reported cases. In 2018, the incidence of cervical cancer ranked second only to breast cancer, with 348,809 reported cases and 207,210 casualties. Indonesia sees an estimated 180,000 new cancer cases annually, with a staggering 75% mortality rate (Agustiansyah et al., 2021). Various treatments have been implemented, including surgery, radiation, synthetic therapies, and the use of herbal medicine, which is typically administered to individuals with tumors (Mosaddad et al., 2021). The advancement of herbal remedies involves rigorous testing through *in vitro*, *in vivo*, and *in silico* methods to scrutinize active compounds and their respective mechanisms of action (Rani et al., 2023).

The anticancer mechanisms, targeting antiproliferation and apoptosis, can be assessed through techniques such as the DNA ladder assay, TUNEL (Terminal deoxynucleotidyl transferase Nick-End Labeling) assay, and comet assay, (Majtnerová & Roušar, 2018), flow cytometer, microscopy perfusion platform, genotoxicity specific chromatin changes (Banfalvi, 2017), as well as immunocytochemistry (Jain et al., 2019). The search for this mechanism was carried out mainly for the development of herbal medicines with various chemical contents.

Many herbal medicines developed as anticancer drugs come from leaves, flower, root and rhizomes (Khan et al., 2020). Rhizomes that have anticancer potential are in the Zingiberaceae family. Research that

has been carried out on this family is *Z. montanum* (Al-Amin et al., 2020), *Z. zerumbet* (Sithara et al., 2018), *Z. ottensii* (Ruttanapattanakul et al., 2021), *Z. cassumunar* (Han et al., 2021), as well as *Z. officinale* (Hasan Mujahid et al., 2023). *Z. officinale* (ZO) has more potential than other zingiber so it is more developed as an anticancer (Syafitri et al., 2018).

Z. officinale is more developed as an anticancer because ZO is a plant with high secondary metabolites and has cytotoxic activity (Nurjannah et al., 2022). *Z. officinale* has been studied *in vitro* on HCT116, B16, MCF-7 (Liao et al., 2020), colon cancer cells (Mohd Yusof et al., 2022), HuH 7 (González-Osuna et al., 2023), and HeLa cells (Hasan Mujahid et al., 2023). The mechanism of action explored in tests on HeLa cells is anti-proliferation using a cytotoxic test with MTT and an antioxidant mechanism with DPPH (Ghazemzadeh et al., 2015). The antiapoptotic mechanism is carried out by Hoest staining (Ansari et al., 2016), and propidium iode staining (Hasan Mujahid et al., 2023). However, from the method that has been conducted, its effect on the cancer cell cycle to the level of the quantity of the cells is unknown. Therefore, assessing the mechanism using the flow cytometry method is required. Flow cytometry results can provide information on the G1 cycle (cell cycle entering the inactive phase). Thus, it is important to utilize a flow cytometer to determine the mechanism of apoptosis orcell death.

An extract is expected possesing anticancer activity ~~using the flow cytometry method~~ when it can stimulate apoptosis as indicated by the number of dead and viable cells as well as the number of cells experiencing apoptosis and necrosis compared with the negative control. Apart from that, the test results with a flow cytometry showed that the sub-G1 phase by sample intervention showed accelerated apoptosis compared to the negative control (McKinnon, 2018). Apoptosis testing with a flow cytometer can be done by staining with Anexin V. The mechanism of apoptosis can be observed from the test results using the FSC-SSC (forward scattered light-Side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) flow cytometry methods (Gadalla et al., 2019).

The next step after testing compound activity is identifying the compounds contained in *Z. officinale* in this regards has anticancer potential with apoptosis mechanism. Essential oils are the dominant compounds in *Z. officinale*. Identification can be done generally using GC/MS. GC/MS analysis of *Z. officinale* has been carried out (Yu et al., 2022). This study was aimed to evaluate the potency of EEZO in inducing apoptosis using the flow cytometry method and to identify its dominant compound that responsible for this effect.

RESEARCH METHODS

Equipment and Materials

The material used is EEZO which is extracted with 75% technical ethanol, aquadest (Bratachem), NaOH (Merck), methanol pa (Merck), HeLa cell culture was provided by Parasitology Laboratory of Faculty of Medicine, Public Health and Nursing UGM, aquabidest, sodium bicarbonate (Sigma), HEPES (Sigma), Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), fetal bovine serum (FBS) 10% v/ v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungizone 0.5% (Gibco), phosphate buffer saline (PBS) 20% (Sigma), dimethyl sulfoxide (DMSO), MTT reagent (3-(4 ,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg in 1 mL PBS (Sigma), stopper, sodium dodecyl sulphate SDS) 10% in 0.01 N HCl (Merck), 96 well-plate, tryptan blue stain 0.25% (Gibco). The equipment used is a CO_2 incubator (New Bronswick, Galaxy 170R), centrifuge (Hermle Siemensstr-25 D-78564), laminar air flow cabinet (Mascotte LH-S, micropipette (Soccorex), autoclave, hemocytometer, ELISA microplate reader (Robonik), microscope inverted (Olympus CKX41-2), GC-MS. EEZO was obtained from extraction using the maceration method and 75% ethanol solvent on *Z. officinale* rhizomes which had been determined from UPT Batu Herbal Materia Medica Laboratory, East Java Provincial Health Service with number 067/1410/102.20/2023.

Research Procedures

7. Flow cytometry test

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The flow cytometry test was carried out on HeLa cells that had been cultured in-depth 96-well plates with a cell density of $5x10^4$ cells/well and were then incubated at 37°C in a CO_2 incubator for 24 hours. The test sample solution (EEZO) was added to the cell culture and incubated in a CO_2 incubator for 24 hours. Next, readings are taken on the flow cytometer with the output settings FSC-SSC, FTIC, and PE in the Pharmacology Laboratory at Faculty of Medicine, Public Health and Nursing UGM.

8. Profiling using GC/MS

The profiling analysis was carried out by dissolving EEZO in a NaOH solution in methanol. A sample of 1 µL was injected with GC conditions: column oven temperature 70.0 °C, injection temperature 280.00 °C, injection mode splitless, sampling time 1 minute, pressure 53.5 kPa, total flow 21.1 mL/min, column flow 0.91 mL/min, linear velocity 34.9 cm/sec, purge flow 2. mL/min, split ratio 20.0, equilibrium time 3.0 min, MS: ion source temp 250.00 °C, interface temp 160.00 °C, detector gain 1.10kV+0.00 kV.

Data Analysis

Flow cytometry results data were analyzed by comparing the flow cytometry results between the control and sample (EEZO). The identification process using the GC-MS tool produces several bioactive compounds that can be seen from the chromatogram peaks for identification. The chromatography results were followed by testing with mass spectrometry (MS) seen from the mass spectrum with each molecular weight of the bioactive compound.

RESULTS AND DISCUSSION

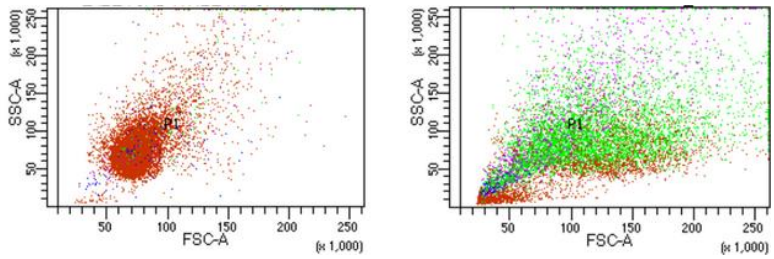
This research was conducted based on ethical clearance from the UAD Ethics Committee number 0123071177. Apoptosis induction testing was carried out on HeLa cells by the influence of EEZO with a flow cytometer by observing at the FSC-SSC, FTIC, and PE images.

3.1. Testing apoptosis with a flow cytometer

3.1.1. *Apoptosis and the flow cytometer FSC-SSC*

Apoptosis can be detected with a flow cytometer based on the number of cells seen as a result of excitation scattering of cells that occurs. This is becouse the scattering produces colors that can be detected in the instrument (Adan et al., 2016). The results of the FSC-SSC flow cytometer image of HeLa cells exposed to EEZO are shown in Figure 1. The cells in the negative control do not appear to be spread out but are homogeneous in the same place at a position that shows the number of live cells. In contrast, the color produced in cells exposed to EEZO showed visible scattering of heterogeneous cell image positions (Figure 1). Apoptotic or necrotic cells may influence these differences in appearance.

The difference in cell images in the FSC-SSC flow cytometer results is because photons when they hit the cell will be deflected around the cell, causing light scattering based on which the FSC and SSC images are formed. FSC is proportional to cell surface area, cell size, nucleus, cell granularity, cell shape, and cell membrane. Furthermore, SSC light is mostly refracted, reflected, and collected which is proportional to the cell granularity or internal complexity which is proportional to the cell type in a heterogeneous population (Adan et al., 2016). The test was carried out at IC_{50} EEZO on HeLa cells so that the cells could be clearly observed in sufficient numbers and was carried out in one test. Cells affected by EEZO appear heterogeneous, which is possible by the presence of live cells, apoptotic cells, and necrosis cells. Meanwhile, the control images show cells in a homogeneous position as the dominant living cells (Figure 1).



(a) (b)

Figure 1. Image of cells from the FSC-SSC flow cytometry of HeLa cells (a) control; (b) treated with *Zingiber officinale* ethanolic extract

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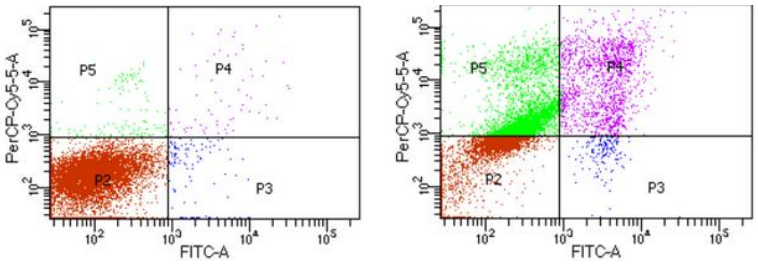
3.1.2. *Apoptosis and the flowcytometer results FTIC*

The results of the FTIC flow cytometer provide an overview of cells in all four quadrants. The lower right quadrant shows the percentage of early apoptotic cells, the lower left shows the percentage of viable cells, and the upper right shows the percentage of late apoptotic cells, the upper left shows the percentage of necrotic cells (Mamat et al., 2021). The FTIC results of HeLa cells in controls compared to those exposed to EEZO have differences, especially in the upper right and lower right quadrants (Figure 2). The right quadrant shows cells undergoing apoptosis. Besides that, EEZO also increased cell death with apoptosis as indicated by the increase in the number of cells seen in the upper left quadrant compared to the control. EEZO was also able to increase cell death as shown in the lower left quadrant, the number of living cells decreased from 9718 cells to 4159 cells (Table 1). Apoptosis and necrosis mechanisms led to the cell death.



(a) **(b)**

Figure 2. **Image of cells from the FTIC flow cytometry of HeLa cells (a) control; (b) treated with *Zingiber officinale* ethanolic extract**

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Table 1. Number of cells in each quadrant of FITC flow cytometer results after treated with *Zingiber officinale* ethanolic extract

Quadrant	Remark	Number of cell	
		Control	EEZO
Bottom left	viable cells	9718	4159
Top right	early apoptosis	109	156
Bottom right	final apoptosis	68	1537
Top left	necrosis	122	4426

3.1.3. *Apoptosis and the flowcytometry results P.E*

The cell cycle that can be observed from the flow cytometry results include the number of cells in the cell growth (G1), synthesis (S), arrest (G2/M) phases, apoptosis in the sub cell growth (sub-G1) phase. Apoptosis can be observed from the results of the sub-G1 phase of the cell cycle from the results of cell counts on the flow cytometer between intervention samples compared to controls. The PE flow cytometer image produces an image of the number of cells entering the sub-G1 phase, where this phase shows cells in the resting phase. The sub-G1 phase can also show cells undergoing the initial apoptosis process (Al-Sheddi et al., 2015). Figure 3 shows that the sub-G1 phase (leftmost quadrant of the figure) of HeLa cells is increased by the influence of EEZO. This increase is in line with the results of FSC-SSC and FTIC which support the occurrence of apoptosis due to the influence of EEZO. This result is confirmed in Table 2 which shows a decrease in G1 (beginning of cell division) from 6799 to 4619 and an increase in sub-G1 from 29 to 68. The results that support the mechanism by which apoptosis occurs are an increase in the cell resting or antiproliferation phase (G2/M) from 1813 to 2318, although the S phase increases from 626 to 849 as a consequence of increasing G2/M. However, the increased synthesis due to EEZO here does not cause the number of cells to increase because it then enters the G2/M phase. This is also supported by the smaller number of cells compared to the control, namely 15744 by the influence of EEZO from the number 18538 in the control. The limitation of this research is the compound that is responsible for the apoptosis mechanism contained in EEZO. However, identifying the dominant compound can provide important information regarding the content of compounds that may have cytotoxic activity from EEZO.

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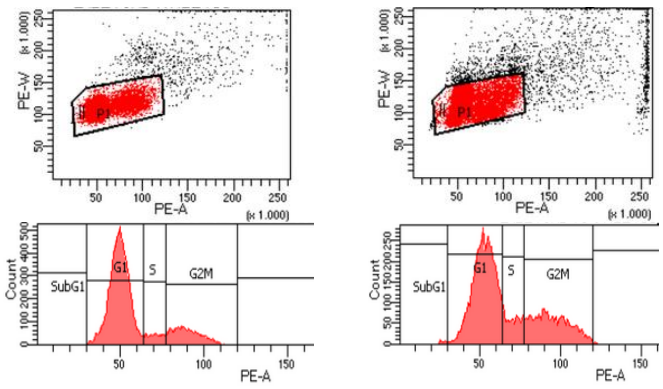

(a) (b)

Figure 3. FSC-SSC, FITC, and PE results from (a) negative control and (b) ethanolic extract of *Zingiber officinale*

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Table 2. Number of cells from PE flow cytometer images of HeLa cells by the influence of *Zingiber officinale* ethanolic extract

Cell phase	Number of Cells	
	Control	EEZO
P1	9269	7872
G1	6799	4619
S	626	849
G2M	1813	2318
Sub-G1	29	68
Polyploid	2	18
Total	18538	15744

3.2. Identification of the phytochemicals in EEZO ~~as an inducer apoptosis~~ using GC/MS

The chromatogram profile and identity of metabolite compounds in the 75% ethanol extract of red ginger rhizomes, the results of analysis using gas chromatography (GC), are presented in Figure 4 and Table 3. The results of the analysis show that there are metabolite compounds that included in the group of terpenoid compounds (monoterpenes and sesquiterpenes), ketones, aldehydes, lipids, and phenols. The metabolite compound profile is an indicator that can be used to provide quality assurance regarding the activity and safety of EEZO as a raw material for natural products (Balekundri & Mannur, 2020; Mattoli et al., 2023).

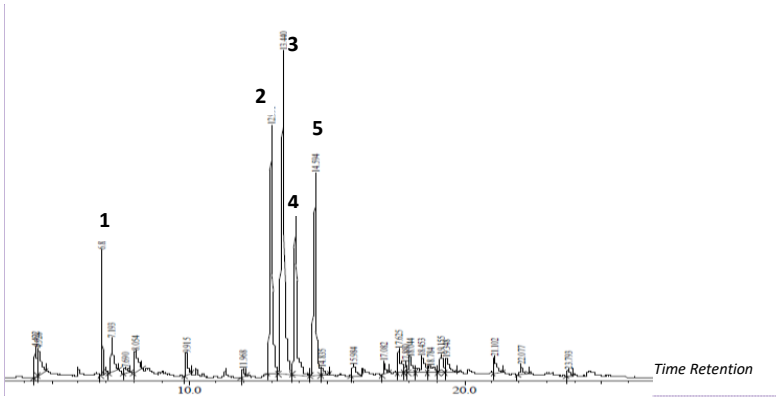

Figure 4. Chromatogram profile of metabolite compounds from *Zingiber officinale* ethanol extract

The chromatogram data in Figure 4 shows that there is the highest peak at a retention time of 13.440 minutes with an area of 25.16% of the total area. These data show the abundance of the compound in EEZO, named zingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene) belongs to the sesquiterpene group of compounds as shown in Table 3. This is in line with the MS results showing the zingiberene compound in Figure 5.

Table 3. Identity of the dominant metabolite compounds of *Zingiber officinale* ethanol extract from analysis using gas chromatography

Peak number	Time retention (minute)	Molecular Weight (m/z)	Molecular Formula	Compound Identity	Compound Classes (ChemIDplus)	Similarity Index (%)
1	6,837	154	$C_{10}H_{18}O$	L-borneol	Monoterpenoids	98
2	12,991	202	$C_{15}H_{22}$	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
3	13,440	204	$C_{15}H_{24}$	zingiberene	Monocyclic Sesquiterpenes	94
4	13,866	222	$C_{15}H_{26}O$	farnesol	Prenols	93
5	14,594	204	$C_{15}H_{24}$	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94

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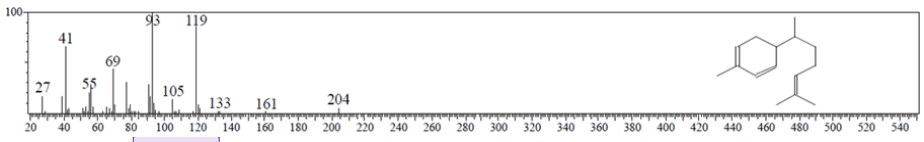

Figure 5. Image of molecular fragmentation on MS of the compound resulting from GC peak number 3 which is identical to zingiberene

Induction of apoptosis in HeLa cells by EEZO is made possible by the active ingredient Zingiberen. Zingiberen can significantly increase the formation of ROS which causes cell apoptosis (Hasan Mujahid et al., 2023) and an increase in sub-G1 in SiHa cells (Lee, 2016). In *vivo* tests were carried out on rats induced by DMBA (Dimethyl benzo anthracene), that zingiberene can act as a chemopreventive (Seshadri, 2022). In addition, Zingiberene can inhibit the growth of colon cancer cells *in vitro* and *in vivo* by inducing autophagy (Chen et al., 2019).

CONCLUSION

EEZO, with its main content the Zingiberene, increases apoptosis in HeLa cells, so it has potential as an anticancer.

ACKNOWLEDGMENT

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The Apoptosis Induction of *Zingiber oficinale* Ethanolic Extract-Treated HeLa (Human Cervical Cancer) Cells and Active Compound Profiling using Gas Chromatography/Mass Spectrometry

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ABSTRACT

Cervical cancer accounts for the highest percentage of cancer-related deaths in Indonesia, comprising nearly 60% of all cases. Research into anticancer mechanisms needs to be carried out. The ethanolic extract of *Zingiber officinale* (EEZO) contains zingiberene, a chemical known for its anticancer activity. Understanding the mechanism behind its apoptosis-inducing effects is crucial. This study aimed to elucidate the apoptotic pathway and analyze the Gas Chromatography /Mass Spectra (GC/MS) profile of EEZO. The research commenced with the maceration of *Zingiber officinale* rhizomes using 75% ethanol to obtain EEZO. Apoptosis assays were conducted on both a negative control group and an EEZO-treated group using HeLa cells (cervical cancer cells). The apoptosis mechanism was evaluated using FSC-SSC (forward scattered light-side scattered light), FTIC (fluorescein isothiocyanate) and PE (phycoerythrin) flow cytometry. Apoptotic results were analyzed by comparing control and EEZO samples, which revealed the number of viable cells, apoptotic cells, and cells in sub-G1 phase. The major constituent in the EEZO that expected having potency as apoptosis inducers were detected using GC/MS. FSC-SSC results indicated a lower number of viable cells in the EEZO-exposed group compared to the control. FTIC results demonstrated that EEZO significantly increased apoptotic cell death, rising from 68 cells to 1537 cells. PE flow cytometry revealed an elevated sub-G1 population, indicating an induction of apoptosis by EEZO. The GC/MS analysis revealed five dominant components in EEZO which had potential as apoptosis inducers were: L-borneol, zingiberene, farnesol, beta-sesquiphellandrene, and alpha-curcumene.. In conclusion, EEZO with its dominant compound, zingeberene, induced apoptosis in HeLa cells and, therefore, indicates anticancer potentials.

Keywords: HeLa cell, anticancer, *Zingiber officinale* (ginger), flow cytometry, zingiberene

INTRODUCTION

Zingiber officinale (ZO) or ginger is a plant with high secondary metabolites and has cytotoxic activity (Nurjannah et al., 2022). ZO has been studied *in vitro* on HCT116, B16, MCF-7 (Liao et al., 2020), colon cancer cells (Mohd Yusof et al., 2022), HuH 7 (González-Osuna et al., 2023), and HeLa cells (Hasan Mujahid et al., 2023). The mechanism of action explored in tests on HeLa cells is anti-proliferation using a cytotoxic test with MTT and an antioxidant mechanism with DPPH (Ghazemzadeh et al., 2015). The antiapoptotic mechanism is carried out by Hoest staining (Ansari et al., 2016), and propidium iodide staining (Hasan Mujahid et al., 2023).

Zingiber officinale contains several bioactive compounds, such as gingerols, shogaols, and zingiberene, which have been shown to induce apoptosis in cancer cells (Lee, 2016). The proapoptotic

effect of ginger is mediated through various signaling pathways with the main pathway being mitochondrial apoptosis. This pathway begins by increasing the expression of the proapoptotic proteins Bax and Bad, while decreasing the expression of antiapoptotic proteins, such as Bcl-2 and Bcl-xL (Mao et al., 2019). This causes the release of proapoptotic proteins, namely cytochrome c, from mitochondria into the cytosol. Cytochrome c binds to apoptosis protease activating factor 1 (APAF1) to form the apoptosome complex, which activates caspase-9. Caspase-9 then activates effector caspases, namely caspase-3 and caspase-7, resulting in apoptosis (Hasan Mujahid et al., 2023).

Gingerol, shogaol, and zingiberene are also known to promote apoptosis through the pathway of inhibiting NF-κB activation by blocking the degradation of IκBα (NF-κBα inhibitor), which prevents NF-κB translocation to the nucleus and subsequent gene expression (Shanmugam et al., 2022).In addition, ZO was found to also stimulate apoptosis by inhibiting the PI3K/Akt/mTOR and MAPK/ERK pathways. This results in reduced cell viability and increased apoptosis in cancer cells (Manna et al., 2020).

The method used to research anticancer mechanisms through apoptosis can be done by observing cells stained with the concept of immunostaining. Observations can be made with an electron microscope, a fluorescence microscope, a time lapse microscope, and an atomic force microscope (Banfalvi, 2017). Before making observations with a microscope, relatively complicated preparations must be made. Moreover, research results cannot directly obtain cell cycles and their numbers quantitatively accurately. Another method for observing apoptosis complete with the cell cycle and quantitative cell counts is the flow cytometer (McKinnon, 2018). However, from the method that has been conducted, its effect on the cancer cell cycle to the level of the quantity of the cells is unknown. Therefore, assessing the mechanism using the flow cytometry method is required. Flow cytometry results can provide information on the G1 cycle (cell cycle entering the inactive phase). Thus, it is important to utilize a flow cytometer to determine the mechanism of apoptosis orcell death. Research on apoptosis of ginger extract with ethanol solvent on HeLa cells using a flow cytometer has never been carried out.

The flow cytometer method is a method used to analyze and sort cells based on their physical and chemical properties, one of which is to observe proapoptotic mechanisms. This method is able to measure the number of cells undergoing apoptosis along with the apoptotic phase. To measure the number of cells undergoing apoptosis, cells are stained with a fluorescent dye that selectively labels cells undergoing apoptosis. Staining with Annexin V resulted in the protein binding to phosphatidylserine, which was externalized on the surface of apoptotic cells. By staining cells with Annexin V and secondary antibodies labeled with fluorescent dyes, identification and size of cells undergoing apoptosis can be identified. The advantage of flow cytometry is that it is a valid, easy, and fast tool to determine the effect of a sample as an anticancer agent using an apoptotic mechanism. Flow cytometry allows the simultaneous analysis of multiple cellular parameters, such as cell size, shape, and fluorescence intensity, making it an ideal technique for studying apoptosis (Crowley, 2016).

In addition to these markers, flow cytometry can also be used to analyze other cellular characteristics such as cell cycle status, surface marker expression, and intracellular signaling pathways involved in apoptosis (Adan et al., 2016). By combining several parameters, flow cytometry is a tool that can explain the apoptotic phase in cancer cells in this study using ZO extract.

An extract is expected possesing anticancer activity when it can stimulate apoptosis as indicated by the number of dead and viable cells as well as the number of cells experiencing apoptosis and necrosis compared with the negative control. Apart from that, the test results with a flow cytometer showed that the sub-G1 phase by sample intervention showed accelerated apoptosis compared to the negative control (McKinnon, 2018). Apoptosis testing with a flow cytometer can be done by staining with anexin V. The mechanism of apoptosis can be observed from the test results using the FSC-SSC (forward scattered light-Side scattered light), FTIC (fluorescein isothiocyanate), and PE (phycoerythrin) fluocy tometry methods (Gadalla et al., 2019).

ZO is a widely used traditional medicine with various health benefits, including anti-inflammatory, antioxidant and anticancer properties. The active compounds responsible for these effects occur in varying types and amounts of compounds in different ginger extracts, and their identification is

essential for understanding the mechanism of action and developing new therapeutic applications. Regarding the type and amount of volatile compounds in ZO, it is very suitable for analysis by Gas Chromatography/Mass Spectrometry (GC/MS) (Nur et al., 2020).

GC/MS analysis allows the separation, identification, and quantification of individual compounds in ginger extracts based on their chemical properties, such as molecular weight, structure, and volatility. GC/MS results can be used to identify chromatograms and spectra which provide information on the basis of retention time and m/z of the compounds contained in them (Nur et al., 2020). This study aims to determine the mechanism of EEZO in inducing apoptosis using the flow cytometry method and to identify its dominant compound that is responsible for this effect.

RESEARCH METHODS

Equipment and Materials

The material used is EEZO which is extracted with 75% technical ethanol, methanol, aquadest (Bratachem), NaOH (Merck), methanol pa (Merck), HeLa cell culture collection from the Parasitology Laboratory Faculty of Medicine Public Health and Nursing UGM, aquabidest, sodium bicarbonate (Sigma), hepes (Sigma), Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), fetal bovine serum (FBS) 10% v/ v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungizone 0.5% (Gibco), phosphate buffer saline (PBS) 20% (Sigma), dimethyl sulfoxide (DMSO), MTT Reagent (3-(4 ,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg in 1 mL PBS (Sigma), stopper, sodium dodecyl sulphate (SDS) 10% in 0.01 N HCl (Merck), 96 well-plate, trytan blue stain 0.25% (Gibco). The equipment used is a CO_2 incubator (New Bronswick, Galaxy 170R), centrifuge (Hermle Siemensstr-25 D-78564), laminar air flow cabinet (Mascotte LH-S), micropipette (Soccorex), autoclave, hemocytometer, ELISA microplate reader (Robonik), microscope inverted (Olympus CKX41-2), GC-MS (QP2010 SE Shimadzu with type of column: Rtx-5MS). EEZO was obtained from extraction using the maceration method and 75% ethanol solvent on *Z. officinale* rhizomes which had been determined from UPT Batu Herbal Materia Medica Laboratory, East Java Provincial Health Service with number 067/1410/102.20/2023.

Research Procedures

9. Flow cytometry test

The flow cytometer test was carried out on HeLa cells that had been cultured in-depth 96-well plates with a cell density of $5x10^4$ cells/well and were then incubated at 37°C in a CO_2 incubator for 24 hours. The test sample solution (EEZO) was added to the cell culture and incubated in a CO_2 incubator for 24 hours. Next, readings are taken on the flow cytometer with the output settings FSC-SSC, FTIC and PE in the Pharmacology Laboratory at Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Yogyakarta.

10.GC/MS analysis

Qualitative analysis using GC-MS was carried out by dissolving EEZO in a NaOH solution in methanol. A sample of 1 µL was injected with GC conditions: column oven temperature 70.0 °C, injection temperature 280.00 °C, injection mode splitless, sampling time 1 minute, pressure 53.5 kPa, total flow 21.1 mL/min, column flow 0.91 mL/min, linear velocity 34.9 cm/sec, purge flow 2. mL/min, split ratio 20.0, equilibrium time 3.0 min, MS: ion source temp 250.00 °C, interface temp 160.00 °C, detector gain 1.10kV+0.00 kV.

Data Analysis

Flow cytometry results data were analyzed by comparing the flow cytometer results between the control and sample (EEZO). The identification process using the GC-MS tool produces several bioactive compounds that can be seen from the chromatogram peaks for identification. The chromatography results were followed by testing with mass spectrometry (MS) seen from the mass spectrum with each molecular

weight of the bioactive compound. The chromatogram obtained was analyzed by comparing the retention time similar to the standard library provided by instrument database. Spectra from GC/MS samples were analyzed by comparing the similarity index (SI) in the instrument database.

RESULTS AND DISCUSSION

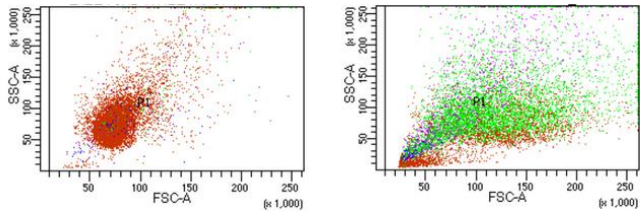
This research was conducted based on ethical clearance from the UAD Ethics Committee number 0123071177. Apoptosis induction testing was carried out on HeLa cells by the influence of EEZO with a flow cytometer by looking at the FSC-SSC, FTIC, and PE images.

3.1. Testing apoptosis with a flowcytometer

3.1.1. *Apoptosis and the flowcytometer FSC-SSC*

Apoptosis can be detected with a flow cytometer based on the number of cells seen as a result of excitation scattering of cells that occurs. This is becouse the scattering produces colors that can be detected in the instrument (Adan et al., 2016). The results of the FSC-SSC flow cytometer image of HeLa cells exposed to EEZO are shown in Figure 1. The cells in the negative control did not appear to be spread out but are homogeneous in the same place at a position that showed the number of viable cells. In contrast, the color produced in cells exposed to EEZO showed visible scattering of heterogeneous cell image positions (Figure 1). Apoptotic or necrotic cells may influence these differences in appearance.

The differences among cell images in the FSC-SSC flow cytometer results were caused by the photons when they hit the cell and being deflected around the cell, causing light scattering based on which the FSC and SSC images were formed. FSC is proportional to cell surface area, cell size, nucleus, cell granularity, cell shape, and cell membrane. On the other hand, SSC light is mostly refracted, reflected, and collected which is proportional to the cell granularity or internal complexity which is proportional to the cell type in a heterogeneous population (Adan et al., 2016). The test was carried out at IC_{50} EEZO on HeLa cells so that the cells could be clearly observed in sufficient numbers and was carried out in one test. Cells affected by EEZO appear heterogeneous, which is possible by the presence of live cells, apoptotic cells, and necrosis cells. Meanwhile, the control images show cells in a homogeneous position as the dominant living cells (Figure 1).



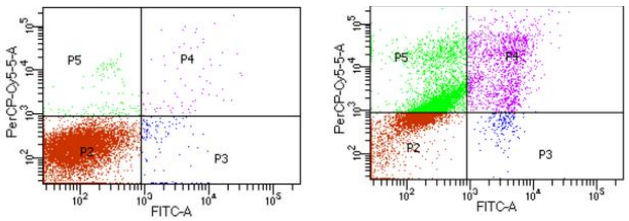
(a) **(b)**

Figure 1. Image of cells from the FSC-SSC flow cytometer of HeLa cells (a) control; (b) treated with *Zingiber officinale* ethanolic extract (●: viable cells; ●: early apopotosis; ●: final apoptosis; ●: necrosis)

3.1.2. *Apoptosis and the flowcytometer results FTIC*

The results of the FTIC flow cytometer provided an overview of cells in all four quadrants. The lower right quadrant showed the percentage of early apoptotic cells, the lower left showed the percentage of viable cells, and the upper right shows the percentage of late apoptotic cells, the upper left showed the percentage of necrotic cells (Mamat et al., 2021). The FTIC results of HeLa cells in controls compared to those exposed to EEZO have differences, especially in the upper right and lower right quadrants (Figure 2). The right quadrant shows cells undergoing apoptosis. Besides that, EEZO also increased cell death with apoptosis as indicated by the increase in the number of cells seen in the upper left quadrant compared to the control. EEZO was also able to increase cell death as shown in the lower left quadrant, the number of living cells

decreased from 9718 cells to 4159 cells (Table I). This cell death process is by apoptosis and necrosis mechanisms.



(a) (b)

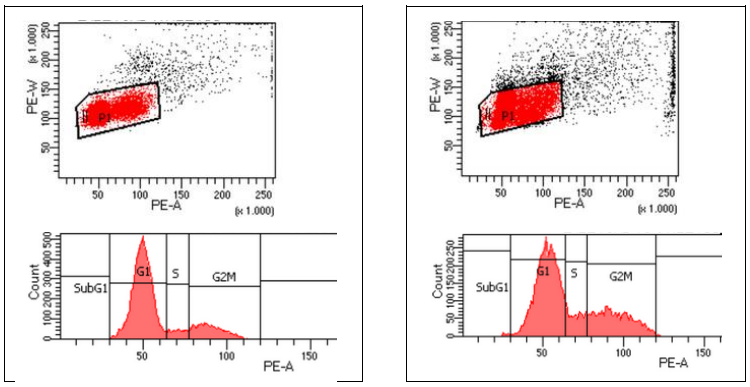
Figure 2. Image of cells from the FTIC flow cytometer of HeLa cells (a) control; (b) treated with *Zingiber officinale* ethanolic extract (●: viable cells; ●: early apopotosis; ●: final apoptosis; ●: necrosis)

Table I. Number of cells in each quadrant of FITC flow cytometer results after *Zingiber officinale* ethanolic extract (EEZO) treatment

Quadrant	Remark	Number of cell	
		Control	EEZO
Bottom left (P2)	viable cells	9718	4159
Top right (P4)	early apoptosis	109	156
Bottom right (P3)	final apoptosis	68	1537
Top left (P1)	necrosis	122	4426

3.1.3. *Apoptosis and the PE flowcytometer results*

The cell cycle that can be observed from the flow cytometry results included the number of cells in the cell growth (G1), synthesis (S), arrest (G2/M) phases, apoptosis in the sub cell growth (sub-G1) phase. Apoptosis could be observed from the results of the sub-G1 phase of the cell cycle from the results of cell counts on the flow cytometer between intervention samples compared to controls. The PE flow cytometer image produced an image of the number of cells entering the sub-G1 phase, where this phase showed cells in the resting phase. The sub-G1 phase could also show cells undergoing the initial apoptosis process (Al-Sheddi et al., 2015). Figure 3 shows that the sub-G1 phase (leftmost quadrant of the figure) of HeLa cells was increased by the influence of EEZO. This increase was in line with the results of FSC-SSC and FTIC which support the occurrence of apoptosis due to the influence of EEZO. This result was confirmed in Table II which showed a decrease in G1 (beginning of cell division) from 6,799 to 4619 and an increase in sub-G1 from 29 to 68. The results that support the mechanism by which apoptosis occurs are an increase in the cell resting or antiproliferation phase (G2/M) from 1813 to 2318, although the S phase increases from 626 to 849 as a consequence of increasing G2/M. However, the increased synthesis due to EEZO here does not cause the number of cells to increase because it then enters the G2/M phase. This is also supported by the smaller number of cells compared to the control, namely 15744 by the influence of EEZO from the number 18538 in the control. In this research, the active compound which takes responsibility to an apoptotic effect has not been revealed. To overcome this limitation, we identified the presence of zingibererene, shigeol and gingerol in ginger extract was identified, although they could not be confirmed as active compounds which act as apoptotic inducer.



(a) (b)

Figure 3. Forward Scattered light-Side Scattered light (FSC-SSC), fluorescein isothiocyanate (FITC), and phycoerythrin (PE) results from (a) negative control and (b) ethanol extract of *Zingiber officinale*

Table II. Number of cells from PE flow cytometer images of HeLa cells by the influence of *Zingiber officinale* ethanol extract

Cell phase	Number of Cells	
	Control	EEZO
P1	9269	7872
G1	6799	4619
S	626	849
G2M	1813	2318
Sub-G1	29	68
Polyploid	2	18
Total	18538	15744

3.2. Identification of the dominant compound in EEZO as an inductor apoptosis using GC/MS

The chromatogram profile and identity of metabolite compounds in the 75% ethanolic extract of ginger rhizomes, the results of analysis using gas chromatography (GC), were presented in Figure 4 and Table III. The results of the analysis showed that there were metabolite compounds that included in the group of terpenoid compounds (monoterpenes and sesquiterpenes), ketones, aldehydes, lipids, and phenols. Sample analysis using GC/MS is used to determine the compound content based on the retention time on the GC and the SI value for structural similarity to standards in the database on the MS instrument (Al-Rubaye et al., 2017).

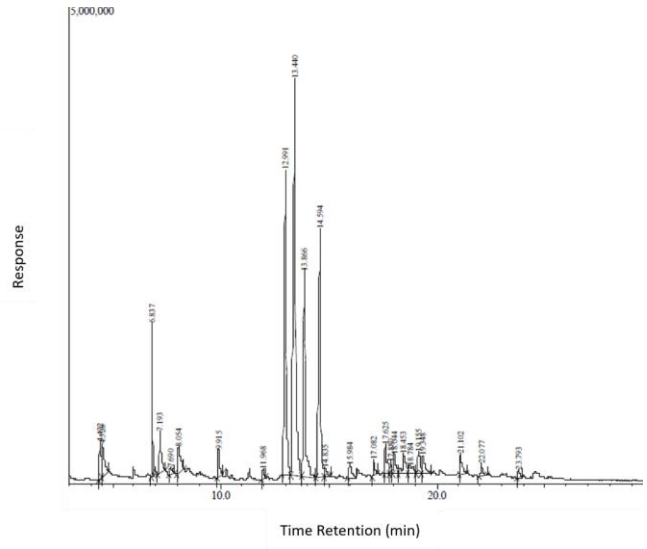


Figure 4. Chromatogram profile of metabolite compounds from *Zingiber officinale* ethanolic extract

The chromatogram data in Figure 4 showed that there was a highest peak at 13.440 minutes with an AUC (area under curve) of 25.16% of the total area of all detected compounds. These data informed the abundance of these compounds in EEZO. The compounds at the retention time were identified as zingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene) belongs to the sesquiterpene group of compounds as shown in Table III. This results were in line with the MS results showing the zingiberene compound in Figure 5. This was based on the results compared with the database in the instrument GC/MS.

Table III. Identity of the dominant metabolite compounds of *Zingiber officinale* ethanolic extract from analysis using gas chromatography

Peak number	Retention time (minute)	Molecular Weight (m/z)	Molecular Formula	Compound Identity	Compound Classes (ChemIDplus)	Similarity Index
1	6.837	154	$C_{10}H_{18}O$	Borneol L	Monoterpenoids	98
2	12.991	202	$C_{15}H_{22}$	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
3	13.440	204	$C_{15}H_{24}$	Zingiberene	Monocyclic Sesquiterpenes	94
4	13.866	222	$C_{15}H_{26}O$	Farnesol	Prenols	93
5	14.594	204	$C_{15}H_{24}$	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94

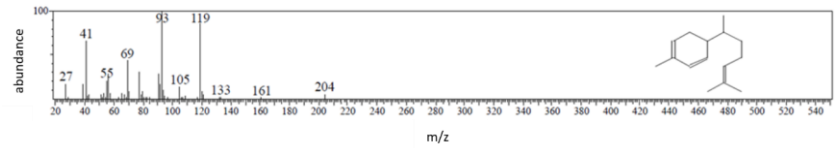


Figure 5. Mass spectrum of the compound resulting from peak number 3. This was estimated to be zingiberene

Induction of apoptosis in HeLa cells by EEZO is made possible by the active ingredient Zingiberen. Zingiberen can significantly increase the formation of ROS which causes cell apoptosis (Hasan Mujahid et al., 2023) and an increase in sub-G1 in SiHa cells (Lee, 2016). In *vivo* tests were carried out on rats induced by DMBA (Dimethyl benzo anthracene), that zingiberene can act as a chemopreventive (Seshadri, 2022). In addition, Zingiberene can inhibit the growth of colon cancer cells *in vitro* and *in vivo* by inducing autophagy (Chen et al., 2019).

CONCLUSION

Based on GC/MS results, EEZO was predicted to contain zingiberene compounds. EEZO potency as an anticancer was confirmed to increase the apoptosis in HeLa cells based on analysis using flowcytometer.

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